An Addition Compound of Oxidized Tocopherol and Linoleic Acid

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ABSTRACT

There is evidence that an addition compound of oxidized *dl*-alpha-tocopherol and linoleic acid is formed when the components are adsorbed in mixed monolayer on silica gel at a molecular ratio of 1:20, and subjected to heating in air at 80 C. A relatively nonpolar tocopheryl quinone is also formed in smaller amounts. These are the major tocopherol oxidation products isolated in this system and do not correspond to any known to the authors. The addition compound has about the same mobility as linoleic acid in most thin layer chromatography (TLC) and chromatographic systems, but can be isolated by successive chromatography on silica and gel filtration on Sephadex LH-20. It yields a single spot in TLC in several systems. The elemental analysis is reproducible and consistent with a simple addition compound of linoleic acid and bivalently oxidized tocopherol. The compound has a carboxyl group which can be esterified. The ester has about the same TLC mobility as methyl linoleate. The molecular weight of the ester is 722.6. The UV spectrum shows a single peak, λ_{max}^{ETOH} = 3000 Å, E = 4.74. The IR spectrum shows a very strong chroman ether band at 9.12 μ, a strong methyl band at 7.24 μ and carboxyl but no hydroxyl absorption. The NMR spectrum shows, in contrast to that of tocopherol, a reduction in aromatic methyl protons, a carboxyl proton exchangeable with deuterium oxide, but no hydroxyl proton. The compound does not reduce Emmerie-Engel reagent prior to treatment with concentrated hydriodic acid, nor do the ether-extractable products after such treatment. The present data are consistent with an addition product whose bridging group is a new chroman ring.

INTRODUCTION

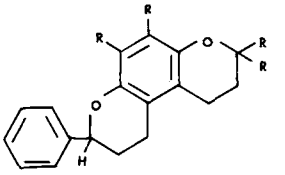
The oxidation of statistical monolayers of fatty acids formed by adsorption on silica gel, both with and without added *dl*-alpha-tocopherol, has been reported from this laboratory (1,2). The products of these oxidations were studied to determine the relative translational mobility of the molecules in the monolayer. Since tocopherol and its oxidation products have characteristic UV absorptions indicative of monomeric and polymeric forms, and since we have shown that the silica gel system, under suitable experimental conditions, permits UV study of the products while in monolayer (2), it was decided to study first the alterations of this molecule. Although quinones and self-polymeric forms have been frequently reported (3-8), compounds of oxidized *dl*-alpha-tocopherol with molecules other than tocopherol under conditions presumably productive of free radicals, have not been frequently demonstrated. Only the 9-hydroxy-alpha-tocopherone (9), the 9-ethoxy-alpha-tocopherone (10), and the 5-benzoyloxymethyl-*dl*-alpha tocopherol (11) appear to have been reported from in vitro reactions. From the alpha-tocopherol model compound, 6-hydroxy-2,2,5,7,8-pentamethyl chroman, Nilsson (12) prepared a styrene Diels-Alder type adduct (Fig. 1) during alkali ferricyanide oxidation in styrene solution. Also from the same model compound, Skinner and Parkhurst (13) have prepared Diels-Alder type adducts with dihydropyran and tetracyanoethylene in aprotic solvents.

About 40% of the tocopherol adsorbed from petroleum ether at a 1:20 molar ratio with linoleic acid in monolayer on silica gel and oxidized at 80 C in air was recovered as a 1:1 adduct of oxidized tocopherol with linoleic acid. The adduct is quite stable, has a free carboxyl group and about the same chromatographic mobility as the free fatty acid in all systems tested, except gel permeation chromatography.

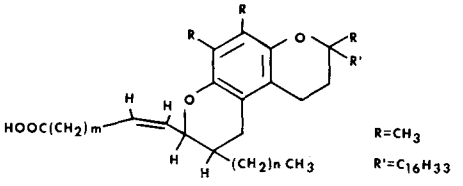
About 10% of the adsorbed tocopherol was recovered as a relatively nonpolar monomeric quinone of tocopherol without a hydroxyl group. Neither of the products corresponds to compounds previously reported (6).

EXPERIMENTAL PROCEDURES

Silica Gel G was supplied by Warner-Chilcott Laboratories Instruments Division, Richmond, California. It was acid-washed four times by the procedure of Stahl (14). Iron content of the final acid washings was 0.75 ppm, as deter-



CHROMANOL-STYRENE ADDUCT



TOCOPHEROL-LINOLEIC ACID ADDUCT

FIG. 1. Structure of chromanol-styrene adduct and of tocopherol-linoleic acid adduct; n + m = 12. Relative location of methyl, carboxyl and double bond of linoleic acid moiety not determined.

mined by atomic adsorption spectrophotometry.

Linoleic acid was purchased from the Hormel Institute, and was used as received, since further purification in our hands gave a product not noticeably more free of UV absorption at 233 mμ than the original, which shows less than 0.1% conjugated diene. There is about 0.1 moles percent of an impurity with the UV spectrum and chromatographic behavior of conjugated trienoic acid.

Both synthetic, racemic *dl*-alpha-tocopherol and natural *d*-alpha-tocopherol were used. The synthetic tocopherol was purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio, and was of N.F. grade (96% tocopherol). It was purified by eight repetitions of the acid and alkali wash procedure of Parker and McFarlane (15). The product gave a single spot in several systems of thin layer chromatography (TLC) and UV absorbance in agreement with literature values.

Natural *d*-alpha-tocopherol was purchased both from Fisher Scientific Company and from Distillation Products Industries. These preparations, which are initially of high purity, were used without further purification from an unopened ampoule and were pure to TLC and UV analysis.

Redistilled, low-boiling (35-52 C) petroleum ether of A.C.S. Reagent Grade was used as adsorbing solvent. It was deoxygenated by 1 hr of flushing with dry nitrogen. Benzene and chloroform used as eluting solvents in chromatography were also of A.C.S. Reagent Grade and were used as received.

Silicic acid used for the initial chromatography was Mallinckrodt A.R. Grade, "Suitable for Chromatographic Analysis by the Method of Ramsey and Patterson." It was activated at 110 C for at least 10 hr, and column beds were washed with about three bed volumes of benzene.

For the final gel filtration of the compounds, Sephadex LH-20 was purchased from Pharmacia Fine Chemicals, Inc. The gel was allowed to swell overnight in a mixture of chloroform and cyclohexane (8:2 v/v). After column packing, it was washed with about three bed volumes of chloroform.

The methods of adsorption on silica gel of a monolayer of lipid containing linoleic acid and tocopherol at a molar ratio of 20:1, subsequent controlled air oxidation at 80 C, and monitoring of the oxidation by oxygen uptake and UV measurements of the monolayer while on silica, have been reported elsewhere (1,2).

Briefly summarized, the method entails adsorption on 2 g of acid-washed, activated Silica G at room temperature of approximately 370 mg of linoleic acid and 25 mg of tocopherol from solution in 45 ml redistilled petroleum ether in an acid-washed, 100 ml round bottom flask. After 40 min of shaking and 3 min of settling, the supernatant solvent is decanted and the adhering residual solvent removed during 1 hr under dry nitrogen flow at slightly above atmospheric pressure. The reaction vessel is then flushed with filtered laboratory air for 5 min, capped with a rubber serum bottle stopper, placed in an 80 C oil bath, and sampled for headspace gas at appropriate intervals, using a Fisher Gas Partitioner (16). After this procedure, no petroleum ether peak could be detected by the Gas Partitioner.

It has been previously shown (1) that adsorption conducted under the above conditions conforms to the Langmuir adsorption isotherm. The amount of linoleic acid adsorbed at the solution concentration used was equivalent to about 75% of theoretical monolayer coverage and was approximately the molecular equivalent of the available oxygen in the flask.

Flasks were removed at appropriate intervals and samples of lipid-coated silica were subjected to successive elution and two-stage chromatography, first in a silica-benzene and later in a gel filtration system. Final purification involved repeated gel filtration, which accomplished the difficult separation of the tocopherol-linoleic acid adduct from linoleic acid.

For the silicic acid column chromatography, since the compounds had been calculated to be in monolayer form on the silica, they were eluted directly from the oxidized lipid-coated

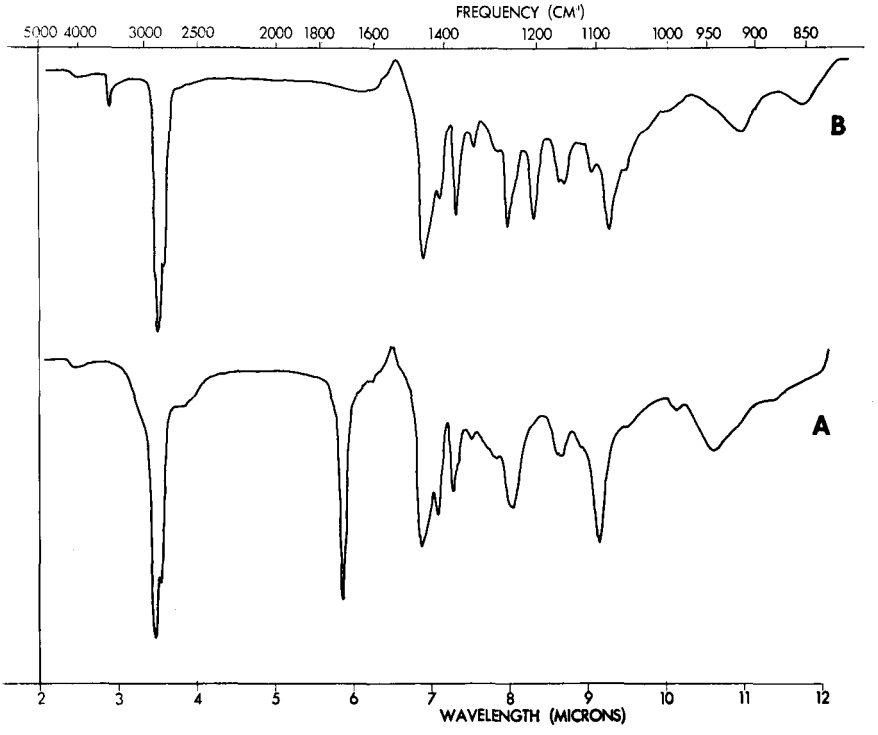


FIG. 2. IR spectrum of tocopherol adduct (A) and of tocopherol (B).

silica onto the top surface of the silicic acid column bed. The 2 g sample of lipid-coated silica, representing the charge of one flask, was dusted through a long-stemmed funnel onto a filter paper disk covering the top of the silicic acid bed surface, which was submerged in ca. 1 ml of the slurrying solvent, benzene. A second disk was floated into place above the charge, solvent was added cautiously, and elution commenced. Among other minor fractions, about 2 mg of a bright yellow, relatively nonpolar tocopheryl quinone was usually eluted at about 1.2 bed volumes. The nearly colorless tocopherol-linoleic acid adduct was eluted at two to three bed volumes, depending on the silica activation. Linoleic acid (and its conjugated trienoic acid impurity) was eluted shortly after the emergence of the adduct, and cochromatographed with it during much of the elution. Of the initial charge of 375 mg, there were retained for gel filtration the middle fractions containing about 165 mg of linoleic acid and 20 mg of tocopherol-derived products.

Since the adduct was highly contaminated with fatty acid after silica chromatography, it was twice rechromatographed by gel filtration on Sephadex LH-20, using chloroform as eluent. In this system, using newly swelled Sephadex, or Sephadex regenerated by chloroform and methanol washing and overnight vacuum drying before swelling, the adduct separates as a pure fraction just preceding the fatty acids, at about two bed volumes.

UV spectrophotometry was performed on a Cary Recording Spectrophotometer, Model 14, using 95% ethanol, unless otherwise specified. IR spectrophotometry was done on a Perkin-Elmer IR Spectrophotometer, Model 21, using CCl_4 as solvent. NMR spectrometry was carried out on a Varian HA-100 NMR Spectrometer. Spectra were recorded at 100 MHz, operating in the frequency-swept mode, using deuterated chloroform as solvent and tetramethylsilane as

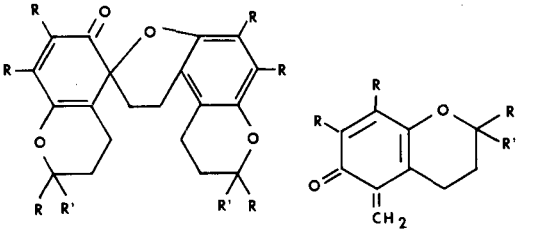


FIG. 3. Structure of tocopherol dimer and of hypothetical quinone methide of tocopherol.

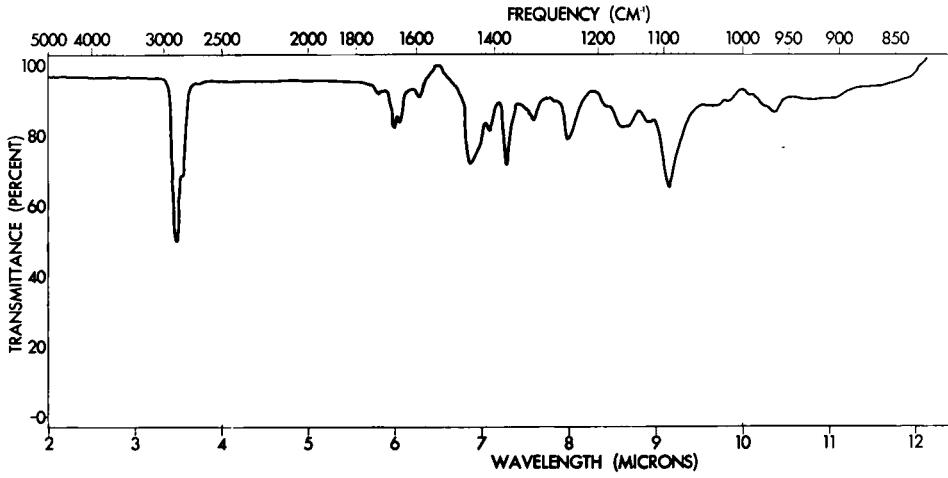


FIG. 4. IR spectrum of tocopherol dimer.

internal standard. Peak values are reported in the tau convention, as parts per million. Mass spectrometry was performed on the methyl ester of the adduct using a CEC Mass Spectrometer, Model 21-110B, with perfluorokerosene as an internal standard and a source temperature of 240 C at time of recording the parent molecular ion. Molecular weight and a confirmation of the elemental analysis were determined by the peak-matching method of Nier (17).

The adduct was characterized by using TLC on Silica G with benzene, esterification with boron trichloride and methanol reagent (18), reaction with the Emmerie-Engel spray reagent and with 58% hydriodic acid (15,19). Analytical data for the compound are shown below.

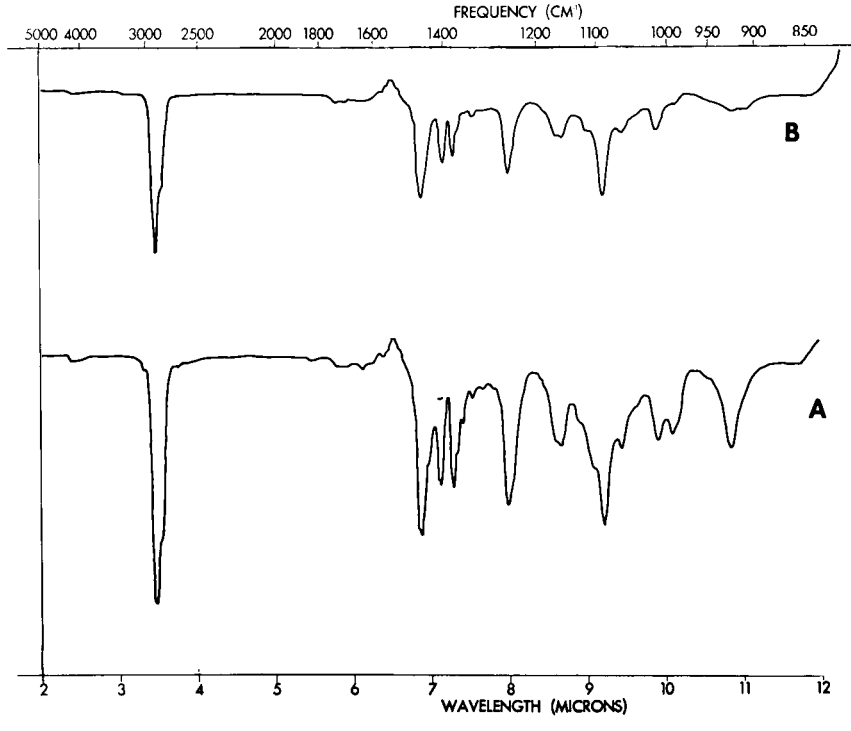


FIG. 5. IR spectrum of tocopheryl allyl ether (A) and of tocopheryl methyl ether (B).

All analytical data on the adduct except that from mass spectrometry were obtained from at least two separately purified samples and were reproducible: pale yellow oil; molecular weight (mass spectrometry) 708.6; R_f linoleic acid 0.07, tocopherol-linoleic acid adduct 0.08, methyl linoleate 0.40, tocopherol-methyl linoleate adduct 0.42; λ_{max}^{ETOH} 300 mμ ($E_{cm}^{g/l}$ 4.74) with blue shift on silica and with polar solvents; IR (μ) carboxyl at 5.84, strong bands at 9.12 (tocopherol chroman) and 7.24 (methyl); NMR, 7.88 (2 aromatic methyl groups), 4.56 (2 olefin protons), 5.93 (1 ether proton), carboxyl but no hydroxyl protons; adduct gives no Emmerie-Engel reaction; with BCl_3-MeOH is converted to an ester giving no Emmerie-Engel reaction; HI treatment does not produce tocopherol or ether-extractable products that give an Emmerie-Engel reaction.

Analysis. Calculated for $C_{47}H_{80}O_4$: C, 79.60; H, 11.37. Found: Sample 1, C, 79.45; H, 12.17. Sample 2, C, 79.32; H, 12.10.

The methyl and allyl ethers of *d*-alpha-tocopherol were prepared, using either methyl iodide or allyl bromide in acetone solution shaken with 36% sodium hydroxide solution at room temperature (20). After two repetitions of silica-benzene chromatography, the middle fractions in both cases were a clear oil for which UV, IR and NMR spectrophotometry were consistent with the relevant ether structure, as was elemental analysis. The ethers were readily cleaved to tocopherol using hydriodic acid (19).

Analysis of allyl ether. Calculated for $C_{32}H_{54}O_2$: C, 81.64; H, 11.56. Found: C, 81.93; H, 11.14. N_D^{20}: 1.4956.

Analysis of methyl ether. Calculated for $C_{30}H_{52}O_2$: C, 81.02; H, 11.79. Found: C, 80.92; H, 11.77. N_D^{20}: 1.4925.

The spiro-keto ether dimer of tocopherol (3) was prepared in over 90% yield using silver oxide. Twenty-two milligrams of *d*-alpha-tocopherol were dissolved in 7 ml of methyl iodide contained in a 50 ml round bottom flask with reflux condenser. One gram of silver oxide was added, whereupon the solution became bright yellow. The suspension was refluxed at 60 C for 3 1/2 hr and filtered. The solvent was removed at reduced pressure to leave 21.5 mg of a bright, orange-yellow oil, soluble with difficulty in ethanol, but very soluble in petroleum ether. The UV absorption spectrum in hexane showed an absorption maximum at 301 mμ and a secondary maximum at 338 mμ ($E_1^{1\%}{}_{cm}$ 5.3, 2.3). This spectrum was very little changed on further purification and closely corresponds to reported values (3-5,7) for the keto-ether dimer of tocopherol. The oil was further purified by column chromatography on silica, using benzene as eluent, the bright yellow oil emerging at 0.79 bed volumes. After two repetitions, the middle fractions of the yellow zone were collected for an analytical sample and were pure to TLC (Silica G-benzene). UV and IR spectra were in agreement with reported values, as was the elemental analysis.

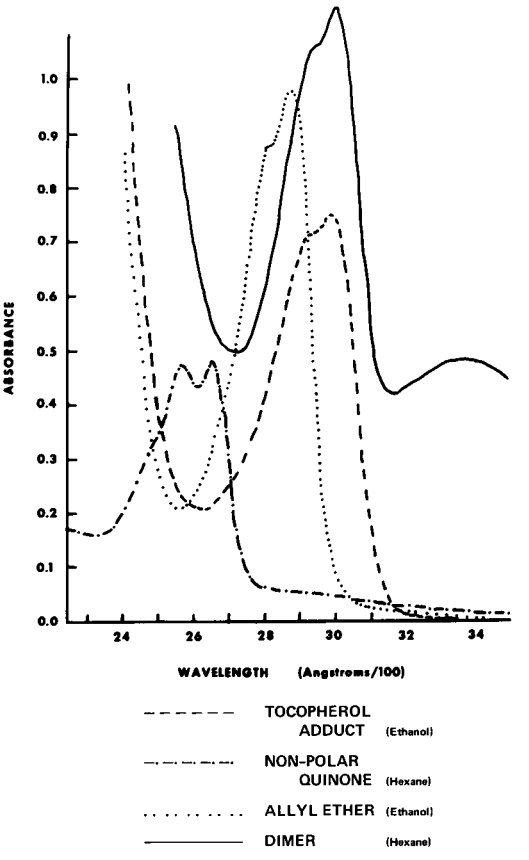


FIG. 6. UV spectra of tocopheryl derivatives. Tocopherol adduct, 0.16 mg/ml. Nonpolar quinone, 0.016 mg/ml. Allyl ether, 0.20 mg/ml. Dimer, 0.21 mg/ml.

Analysis. Calculated for $C_{58}H_{96}O_4$: C, 81.25; H, 11.29. Found: C, 80.62; H, 11.26.

RESULTS AND DISCUSSION

Course of Oxidation in the Linoleic Acid-Tocopherol Monolayer

It was found, using silica-UV and gravimetric measurement methods previously described (1,2), that about 4.3 moles per cent tocopherol were co-adsorbed with 185 mg of linoleic acid in a monolayer on 1 g of silica from 25 ml of petroleum ether solution initially containing 10 mg/ml of linoleic acid and 0.8 mg/ml of toco-

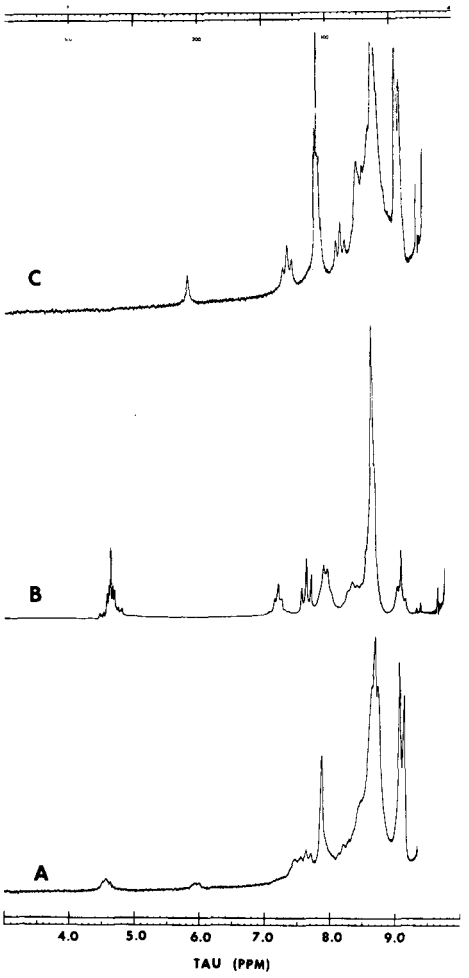


FIG. 7. NMR spectrum of tocopherol adduct (A), linoleic acid (B) and tocopherol (C).

pherol. In the typical experiment at this concentration and at a temperature of 80 ± 2 C, oxygen uptake was followed for five to seven days, or until headspace oxygen had reached less than 1%. This oxygen consumption was approximately equivalent to 1 mole of oxygen per mole of linoleic acid.

The course of oxygen uptake and the changes in the UV spectrum of such a monolayer on silica have been reported (1). Briefly summarized, during the first day of a four day "induction period," there is a pronounced uptake of oxygen (10% of available oxygen), coincident with a shift in the peak of maximum UV absorbance from 285 mμ (tocopherol adsorbed on silica) to 277 mμ (quinone adsorbed on silica). The oxygen consumption then declines slowly and the UV spectrum remains nearly constant for three more days. However, at the

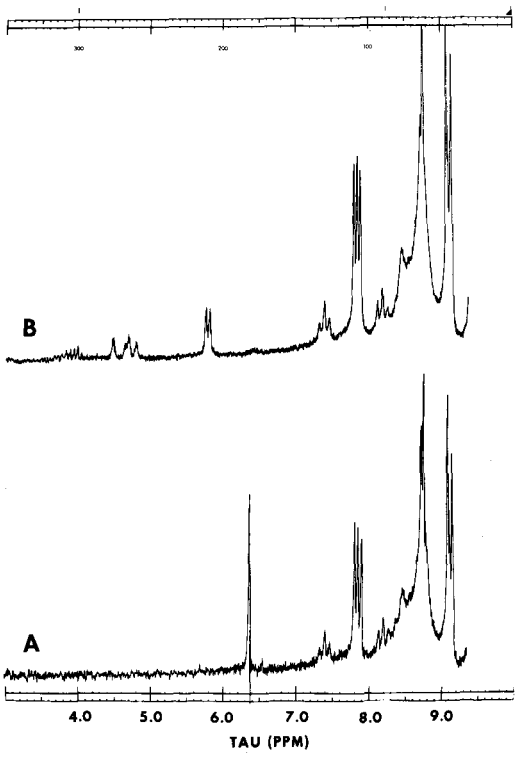


FIG. 8. NMR spectrum of tocopheryl methyl ether (A) and of tocopheryl allyl ether (B).

end of the fourth day about 25% of the available oxygen has been consumed, or about 5 mole/mole of tocopherol. After this four-day tocopherol-dependent "induction period," there is a sharp increase in oxygen consumption and an abrupt shift in the peak of the silica-UV spectrum back to 283 mμ (diene carbonyl on silica). Almost all of the oxygen is consumed within the next day or two.

Oxidation Products Derived From Tocopherol

Silicic acid chromatography of the products of oxidation direct from the monolayer on silica just prior to the end of the four-day "induction period" showed two predominant products traceable to tocopherol. A yellow, relatively non-polar, monomeric quinone without a hydroxyl group (Compound A) is eluted after about one bed volume of benzene as eluent. The UV absorption spectrum in ethanol showed maxima at 261, 267 mμ ($E_1^{1\%}{}_{cm}$ approx. 300). Compound B, a nearly colorless oil, appears after three or four bed volumes, just prior to linoleic acid and, in later fractions, merged with it. It is a tocopherol-linoleic acid adduct, the analytical characteristics of which have been described above.

These two compounds, at most, represent about 50% of the available tocopherol. Neither silica-UV spectrophotometry nor subsequent chromatography gave evidence for other monomers, dimers or trimers as elsewhere reported from tocopherol oxidation.

The Tocopherol-Linoleic Acid Adduct

Because it is present in larger amounts, and because of its possible importance to tocopherol function, the adduct was the more thoroughly characterized of the two products. Many of its characteristics have been stated above, but some should be discussed in more detail.

The elemental analysis most closely corresponds to that calculated for a simple or multiple adduct of the composition: linoleic acid + tocopherol, $C_{47}H_{82}O_4$. However, the parent ion molecular weight derived from mass spectral data for the ester, 722.617, corresponds more closely to that calculated for $C_{48}H_{82}O_4$, 722.621, consistent with methyl linoleate + tocopherol - 2H, the coupling of bivalently oxidized tocopherol with methyl linoleate (Fig. 1 for the acid).

The IR spectrum (Fig. 2) shows the presence of a carboxyl group at 5.83 μ and the methyl (7.24 μ) and chroman (9.12 μ) groups of tocopherol but no hydroxyl or unsaturated carbonyl groups. The chroman peak arises from the CO stretch vibration of the cyclic aromatic ether ring. The ratio of the absorbance of the methyl-methylene peak at 3.42 μ to that of the carboxyl group is 1.50 for the adduct, compared to 0.78 for linoleic acid alone, nearly a twofold increase, as expected for the hypothesized adduct.

In general, then, the IR spectrum confirms the presence of both linoleic acid and tocopherol moieties. Only one oxidation product of tocopherol is known which has an esterifiable carboxyl group (21). The ring of this compound, alpha-tocopheronic acid, has a quinone structure and reactions, which Compound B, the adduct, does not. The chroman peak of the adduct is twice as intense as that in the tocopherol spectrum, and its wave length matches that of the chroman peak of the known tocopherol dimer (Fig. 3,4). The dimer contains a second chroman ring linking the monomers (4,8) and is probably derived from a quinone methide intermediate. The absorption by the adduct in the acyclic aromatic ether region around 8 μ is weak in contrast to the comparable absorption of the allyl and methyl tocopheryl ethers (Fig. 5).

Of the four oxygens in the 1:1 adduct suggested by the elemental analysis and mass spectrometry, the IR analysis permits assignment of two to the carboxyl and one to the chroman group. The assignment of the remaining oxygen is critical to the problem of structure. The IR analysis and the failure of the adduct to give an Emmerie-Engel reaction rule out hydroxyl and unsaturated carbonyl groups. Thus, for the group containing the remaining one atom of oxygen, there remain only simple or cyclic ethers as alternatives, either of which places a requirement that the aromatic structure be preserved, since no ether peak other than that of the intensified chroman is observed in the 9 μ cyclic and aliphatic ether region.

The UV spectrum in ethanol (Fig. 6) adds strong support to the aromatic ether assignment. The main 300 mμ band shows a slight bathochromic shift to 301 mμ in less polar solvents like chloroform, and a pronounced hypsochromic shift in more polar systems such as the silica-UV system (2), where the peak is at 295 mμ. We have found such a hypsochromic shift in polar media to be associated with the aromatic ring of all tocopherol derivatives so far examined, whenever aromaticity is preserved. Thus, tocopherol and its allyl and methyl ethers, and the main 300 mμ band of the dimer, which contains one aromatic ring, shift in this manner. On the other hand, tocopherol quinones experience a bathochromic shift on silica of 7-10 mμ, and the unsaturated ketones, in particular the ketone band of the dimer, also shift bathochromically, often as much as 30 mμ. Pure conjugated polyene systems of up to three double bonds shift only slightly bathochromically.

Confirmation of aromaticity was provided by the NMR spectrum. In addition to the usual methyl peaks between 9.06-9.12 τ and methylene peaks between 8.63-8.73 τ, representing the aliphatic chains of linoleic acid and tocopherol (Fig. 7), there occurred broad, asymmetric peaks at 4.56 τ and 5.93 τ, which were equivalent to two and one protons, respectively. The former peak is in the region of nonconjugated olefinic protons, while the latter is not exchangeable with deuterium oxide, and is in the region of protons on carbon alpha to an aromatic ether oxygen. A carboxyl proton exchangeable with deuterium oxide was located below the zero of the scale. The most prominent peak below 8.0 τ was the strong singlet at 7.88 τ, representing six protons of two tocopherol methyl groups. This low field position is indicative of methyl groups attached to aromatic or olefinic carbons (22).

The assignment most critical to structure is that of the 7.88 τ singlet, since it must arise from the ring methyl groups of tocopherol (Fig.

7), which have no adjacent alpha carbon with a proton in any known structures. Since it has been shown by the IR that there is no unsaturated carbonyl function in the ring, it appears confirmed that the ring remains aromatic, and that the single unassigned oxygen atom is part of a simple or cyclic aromatic ether link. Strong support for this assignment is found in the peak at 5.93 τ, which is in the region of protons on carbon alpha to an aromatic ether oxygen, as is evident in the spectra of the allyl and methyl ethers (Fig. 8). This peak in the adduct is equivalent to a single proton, which suggests that the parent carbon atom is secondary, and thus, part of a chain.

It appears, however, from an inspection of the NMR spectra of the allyl and methyl ethers of tocopherol that the adduct does not have a simple aromatic ether link. Unlike the adduct, both of these ethers with widely differing oxygen substituents show a characteristic benzylic methylene triplet at 7.4 τ, nearly the same as that in tocopherol, and a very distinct triple singlet structure of the aromatic methyls in the 7.85 τ region. In addition, both of these ethers have a UV absorption maximum at about 288 mμ in ethanol, in contrast to 300 mμ for the adduct and the main band of the dimer.

The failure of hydriodic acid treatment to produce ether-extractable compounds reacting like tocopherol in the Emmerie-Engel reaction is further evidence that a simple aromatic ether bridge is not involved, since the simple allyl and methyl ethers readily produce tocopherol under these conditions.

All the above data, the similarity of the IR spectrum in the 8-9 μ region to that of the tocopherol dimer (Fig. 3,4), (to which has been assigned the structure of a second chroman ring as a bridge) and Nilsson's demonstration (12) of a styrene-chromanol adduct for which he postulates a second chroman ring bridge (Fig. 1), appear consistent with an adduct whose bridging group is a new chroman ring.

The present findings of a monomeric, non-hydroxylic quinone, and an adduct of oxidized tocopherol and linoleic acid, coupled with failure to find self-polymeric forms of oxidized tocopherol reported by other workers from one- and two-phase bulk liquid systems, suggest that tocopherol oxidation follows a different course in the silica monolayer system. Thus, tocopherol molecules appear to be prevented, either by separation or by steric restrictions, from mutual association and reaction. However, the existence of the adduct in proportionately large yield suggests that the chemical reactivity of the tocopherol molecule with other molecules is still considerable. The data are thus consistent with a relatively unrestricted reaction rate, but a greatly reduced migrational mobility. Since tocopherol is the more mobile of the two components in most silica chromatographic systems, it would seem plausible that both components are quite firmly anchored, that their initial distribution is quite random, with little tendency for segregation of the two components, and that the auto-oxidation reaction may be propagated in domino fashion, with little lateral translation.

If these findings are not unique to the system described, analogous compounds may be demonstrable using membrane constituents like unsaturated phospholipids on silica, or in lyophilized, or in vivo tissue systems.

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A Method of Ultraviolet Spectrophotometry of Lipid Monolayers on Silica Gel

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ABSTRACT

A method has been developed for visible and UV spectrophotometry of lipid monolayers after preadsorption on silica gel from solution in hydrocarbon solvents. Lipid-coated silica gel is made optically transparent in the desired spectral region by slurrying with an inert solvent mixture. Reproducible gels with nearly the same refractive index as the solvent are achieved by careful choice of mixed solvents and carefully timed settling periods. The gel is pipetted into a silica cuvette of 1 cm light path, and a 9 mm silica spacer bar is inserted between Teflon guide strips. Appropriate choice of solvent mixtures (mainly spectral grade cyclohexane and cyclooctane) permits quantitation of spectra down to 2250 Angstrom units, using a silica gel reference cell. Lipid elution from the monolayer into the solvent is usually less than 1% at the dry-mixing dilutions used. Spectra of known unsaturated carbonyl compounds adsorbed on silica show red shifts as great as 240 Angstrom units, while blue shifts as great as 100 Angstrom units are observed for tocopherol derivatives with an intact aromatic ring. Very small shifts are observed for polyenes. The extent and direction of the shifts are indicative of adsorption and its orientation and are useful in the preliminary determination of the class of an unknown compound. The silica slurry spectra of the important tocopherol oxidation products, the quinone, dimer and trimer, have been examined. Peak wavelength shifts are consistent with structures elsewhere proposed for these compounds.

INTRODUCTION

To study the autoxidation of monolayers of linoleic acid adsorbed from solution at equilibrium onto silica gels of high specific area, it was necessary to develop a method of studying the products while still in the monolayer. This would reduce the risk of confusion caused by artifact formation during elution from the monolayer. Also, intermediate products, whose survival depends upon stearic or mobility restrictions prevailing in the monolayer, could be detected.

Because of the high sensitivity of UV spectrophotometry and the fact that silica gel transmits UV light down to 2200A, the products were studied by UV spectrophotometry using a gel of the lipid-coated silica in a solvent of approximately the same refractive index as the silica at the wavelength region desired. Scattering effects are minimized by this procedure, although they cannot be eliminated because of the differing refractive indices and dispersions of the lipids, their oxidized products and the silica.

Leermakers and coworkers (1-4) have made the most recent studies on the spectra and photochemistry of adsorbed organic molecules using silica gel in a quartz cell of path length 0.1 cm, added with stirring to a solution of the compound to be studied at appropriate concentration in cyclohexane. Robin and Trueblood (5) studied the spectra of organic molecules adsorbed on silicic acid in cyclohexane slurries previously prepared with magnetic stirring and poured as an aliquot into a quartz cell with path length 0.3 cm. Because of the close proximity of acidic protons of the silanol groups of silicic acid, an intense electrostatic and hydrogen-bonding field seems to exist at the surface. Thus, pronounced spectral changes both in peak wavelengths and in molar absorptivity may result from adsorption. Leermakers states, "In general, red shifts occur on adsorption of a compound onto the polar adsorbent silica gel if the excited state of the molecule has an increased permanent dipole or if it is more polarizable than the ground state; the blue shifts occur if the reverse is true" (1). The extent and direction of the shifts indicate the fact of adsorption, the nature of the adsorbing group in the molecule and, to a degree, the orientation of adsorption.

EXPERIMENTAL PROCEDURES

Materials

Purifications of Silica Gel G, tocopherol and petroleum ether used in adsorbing monolayers of lipid and reference compounds have been

described elsewhere (6). Neutral alumina (Brockman Activity 1) was purchased from Fisher Scientific Co.

Solvents for silica slurry spectrophotometry were appropriate mixtures of cyclohexane and cyclooctane. The cyclohexane was of spectral grade, purchased from Matheson, Coleman and Bell. Cyclooctane was purchased from Eastman Organic Chemicals, Rochester, N.Y., and Colombian Carbon Company, Princeton, N.J. The cyclooctane was not of spectral grade and contained an impurity adsorbing between 2600 and 2700 A. This impurity was reduced to approximately one fifth by shaking three times with one half the volume of concentrated sulfuric acid, followed by washes with water and saturated sodium bicarbonate until the effluent was neutral. The product was then dried over magnesium sulfate. Thus purified, it was suitable for silica gel spectrophotometry down to 2100 A.

Crotonaldehyde was procured from Eastman Organic Chemicals (Rochester, N.Y.). 2,4-Hexadienal and 2,4-hexadienol were procured from Aldrich Chemical Co., Milwaukee, Wisc. Mesityl oxide was procured from Matheson, Coleman and Bell. Eleostearic acid and ethylidene acetone were procured from Pfaltz and Bauer, Inc., Flushing, N.Y. The *d*-alpha tocopheryl quinone was procured from Distillation Products Industries, Rochester, N.Y.

The volatile materials were purified by redistillation. Tocopheryl quinone was purified by column chromatography on silica and gave a single spot in thin layer chromatography. UV absorption values of both tocopheryl quinone and eleostearic acid agreed with those found in literature.

Preparation of the spiro-keto ether dimer and of the allyl and methyl ethers of tocopherol is described elsewhere (7). The trimer of tocopherol was prepared by standard procedures (8), except that final purification was by two repetitions of column chromatography on neutral alumina, using petroleum ether-diethyl ether mixtures, the final elution being with petroleum ether-diethyl ether (19:1) which separates trimer A from trimer B. The characteristics of trimer A, including elemental analysis, were in agreement with values found in literature, with the exception that the IR spectrum (CCl_4) showed a strong 5.93 μ peak, as reported, but neither trimer A nor B showed a 5.80 μ peak.

The method of adsorption of lipids and reference compounds on activated Silica Gel G from petroleum ether solution is described elsewhere (6).

Silica Slurry UV Spectrophotometry

UV spectrophotometry of the lipid-coated silica was conducted in standard silica cuvettes of 1 cm path length and of approximately 4 ml capacity. A reproducible and optically homogeneous slurry of the coated silica was prepared in cyclooctane and cyclohexane solvent mixtures, placed in the bottom of the cuvette, and extruded with a 9 mm quartz spacer bar inserted rapidly into the gel to produce two bubble free, homogeneous layers of slurry totaling 1 mm in path length. Evaporation of the volatile solvent mixture was prevented by a cap of "Saran Wrap" secured with a small rubber band placed over the spacer bar and cell. A reference sample was prepared using uncoated, acid-washed, heat-activated silica in a solvent slurry in a similar cell and spacer combination.

To prepare the silica for UV spectrophotometry a weighed aliquot (about 100 mg) of the coated silica containing autoxidized lipid, which was kept under dry nitrogen at O C, was carefully and quickly weighed after restoration to room temperature under dry nitrogen. At the same time, sufficient acid-washed, heat-activated, uncoated silica (about 900 mg) was mixed with the coated silica to compose 1 g. The coated and uncoated silicas were dry-mixed for 5 min in a 50 ml round bottom flask with a small magnetic stirrer bar by manual shaking under dry nitrogen flush at slightly above atmospheric pressure. An escape manifold provided a visual monitor of silica loss, which was kept to a minimum consistent with a steady nitrogen flow.

To form the slurry, exactly 3 ml of a suitable solvent mixture (typically cyclooctane-cyclohexane, 2:8 v/v) was pipetted directly into the bottom of a 25 ml graduated cylinder (19.5 x 1.5 cm) with standard taper orifice and glass stopper, taking care not to wet the walls. A 12 mm magnetic stirrer bar was added and a long stem filter funnel was inserted into the graduate with the stem of a length sufficient to nearly touch the liquid. The mixed silicas or, alternatively, the blank silica, were slowly added with stirring by funnel so that each increment was wetted and incorporated into the slurry without forming a dry powder on the walls of the cylinder. The slurry formed in this manner was almost immediately optically homogeneous; there were no visible bubbles and very little slurry on the walls of the cylinder. At this time, when no clear supernatant was visible, stirring was stopped, the cylinder was stoppered, and the slurry was allowed to settle for a 3 min interval which was carefully timed by a laboratory timer with an

TABLE I

Spectral Shifts of Reference Compounds on Silica

Compound	Wavelength of maximum absorption[a] A units: Cyclohexane-cyclooctane solution (8:2 v/v)	Wavelength of maximum absorption[a] A units: Silica gel-cyclohexane-cyclooctane (8:2 v/v) slurry	Spectral shift, cm^{-1}
Crotonaldehyde	Below 2200	2270	--- (red)
Ethylidene acetone	Below 2200	2295	--- (red)
Mesityl oxide	2315	2470	2711 (red)
Sorbaldehyde	2630	2870	3180 (red)
D-alpha-tocopheryl quinone	2610-2695	2680-2755	808 (red)
Sorbyl alcohol	2295	2295	0 (none)
Beta-eleostearic acid	2705	2720	204 (red)
D-alpha-tocopherol	2980	2880	1165 (blue)
Tocopheryl allyl ether	2880	2850	365 (blue)
Tocopheryl methyl ether	2880	2850	365 (blue)
Keto ether dimer of tocopherol	3020	2950	786 (blue)
	3410	3770	2800 (red)
Trimer of tocopherol	2945	2910	408 (blue)
	---	2525	--- (red)

[a]Based on slant baseline. Method described in text.

alarm. The reference slurry was mixed first; after it had settled approximately 1½ min, the 3 min settling period for the coated sample was begun and timed in the same manner.

At the end of the timed period, the volume of the slurry was 3.25 ml. An 0.8 ml aliquot of the slurry was slowly and carefully withdrawn from a position 2 mm above the bottom of the graduate using a finger-controlled pipette filler and a pipette prepared from a fire-polished glass tube 4 mm i.d., scratch-marked at a suitable point. Seven tenths of a milliliter of the slurry sample was deposited in the open cuvette at a point 2 mm from the bottom and the 9 mm quartz spacer bar was thrust quickly and firmly into the slurry, extruding it up the cell walls. To prevent the development of cracks in the slurry, it was found that this operation must be performed quickly and preferably by two people. The Saran Wrap cell cover was secured, the cell surfaces gently wiped, and the cell placed in the spectrophotometer cuvette holder. The effective life of such a preparation is approximately 10 min before cracks and bubbles develop. Therefore, a calibration baseline was made using the cuvettes alone or filled with liquid solvent instead of slurry. Experience had proven the adequacy of such a procedure for precision; the rapidity of onset of deterioration of the reference slurry prevented its use against both a blank and the sample. The deterioration is relatively sudden and immediately detectable by eye. Prior to this, repetitions of optical absorbance never differ more than 5%, and changes in the position of the peak wavelengths are not observable.

Spectra were read on a Cary Recording Spectrophotometer, Model 14M, using a slit control setting of 50 and full slit height. Under the above conditions, spectra could be obtained between 2250 and 3600 A using the 2/8 cyclooctane-cyclohexane solvent mixture, the shorter wavelength limit being set by the absorbance of the reference silica and the consequent increase in slit width. In general, an initial preparation was followed by a repeat preparation of both sample and reference slurries. Optical repetitions on the same slurry differed so little that they were not included in the standard procedure. Preparative repetitions from the same dry mixture of silica in general differ less than 5% in absorbance, and even when preparations are made from separately weighed and mixed silica samples, the cumulative variability in molar absorptivity is less than 10%. The wavelengths of maximum absorption are very reproducible, particularly above 2400 A, but errors inherent in slurry preparation and settling permit less precision for molar absorptivities.

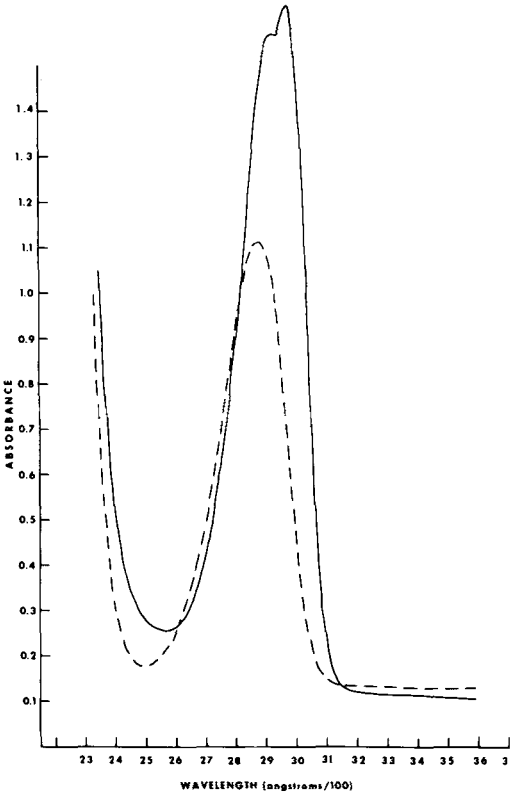

FIG. 1. UV spectrum of d-alpha-tocopherol. Cyclooctane-cyclohexane, 2:8 (solid line); cyclooctane-cyclohexane-silica slurry (dashed line); 0.176 mg/ml.

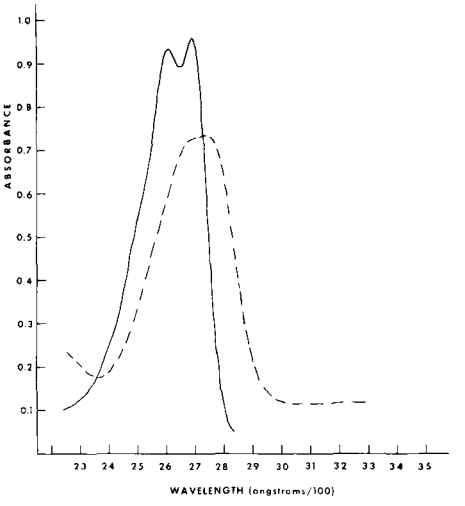

FIG. 2. UV spectrum of d-alpha-tocopheryl quinone. Cyclooctane-cyclohexane, 2:8 (solid line); cyclooctane-cyclohexane-silica slurry (dashed line); 0.023 mg/ml.

RESULTS AND DISCUSSION

Selection of Slurrying Solvent

Very few solvents are suitable for performing UV spectrophotometry with silica precoated with lipid monolayers. They cannot have any important UV absorption above 2100 A. They must be relatively inert chemically, and, preferably, aliphatic hydrocarbons. More polar materials will strongly elute products from the silica surface. Solvents must be relatively nonvolatile and possess an index of refraction high enough to match the lipid-coated silica in the ranges desired. The components of a solvent pair must be suitably different in index so that matching mixtures may be found for the desired range. It is also important that the supernatant be clear, to insure that all the coated silica particles are being included in the measured volume after settling of the slurry. More polar solvents fail to produce such coherence of the slurry. The cyclooctane-cyclohexane combination in various proportions was found to be the best.

Optical Limitations of the Method

To increase transparency of oxidized lipid-coated silica slurries in the 2200 to 2500 A regions where diene hydroperoxide and alpha-beta unsaturated carbonyl compounds absorb, cyclooctane-cyclohexane mixtures were used, rather than cyclohexane alone (Robin and Trueblood, and Leermakers). However, any spectrophotometric studies of silica solvent slurries are limited by the fact that the refractive dispersions (increase of refractive index with decreasing wavelength) of liquids are generally greater than those of solids. A good match at one wavelength is an imperfect match at another wavelength, i.e., a slurry nearly transparent in the UV may be only translucent in the visible range. This is responsible for the well known Christiansen-filter effect of enhanced transmission of a slurry in the range of best match of solvent and solid indices of refraction (5). To this is added the effect of the small particle size of the silica, which always tends to increase Rayleigh scattering toward lower wavelengths. Furthermore, the lipid which is in the monolayer coating on the silica usually has a different index of refraction from either the silica or the solvent, and this difference is strongly enhanced upon oxidation. Thus a perfect match of solvent and coated solid, even at one wavelength, is impossible, and a compromise is necessary in choosing solvent mixtures for the particular lipid material and wavelength region of study. In addition, a solvent refractive index which matches the refractive index of the uncoated reference

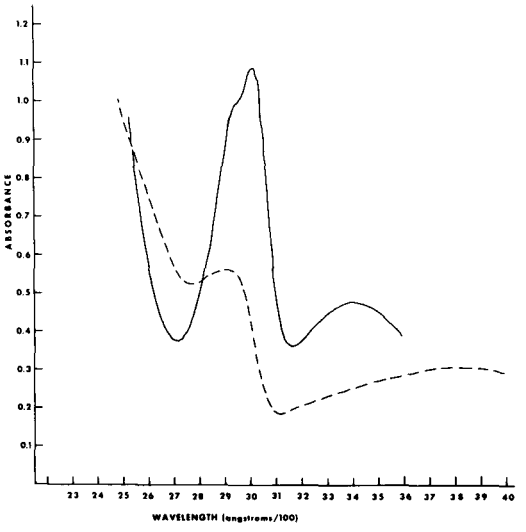

FIG. 3. UV spectrum of keto-ether dimer of d-alpha-tocopherol. Cyclooctane-cyclohexane, 2:8 (solid line, 0.192 mg/ml). Cyclooctane-cyclohexane-silica slurry (dashed line, 0.128 mg/ml).

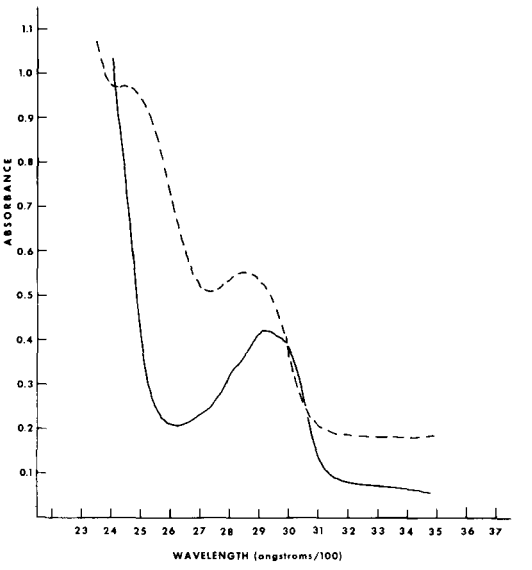

FIG. 4. UV spectrum of tocopherol trimer A. Cyclooctane-cyclohexane, 2:8 (solid line); cyclooctane-cyclohexane-silica slurry (dashed line); 0.086 mg/ml.

silica, and hence extends the spectral range to lower wavelengths, may add to the scattering background of the coated silica sample which appears like an "end-absorption" or a slowly rising background toward shorter wavelengths.

The extinction of uncoated silica slurries, prepared as above in cyclooctane-cyclohexane (2:8), showed the following values at representative wavelengths: wavelength 2300 A, 1.5 extinction; 2900 A, 0.4; 3600 A, 0.5.

Theoretical maximum transmission or the Christiansen "window" (3) should occur nearer to 2300 A, but the nonselective increase of Rayleigh scattering with decreasing wavelength shifts this point toward the red. In general, however, the optical density of these slurries is low, compared to the dense, light-scattering materials for which Butler (9) reports selective wavelength-dependent scattering due to anomalous dispersion in the region of absorption bands. Anomalies due to stray light and fluorescence found in dense, light-scattering materials are also minimized by the much lower optical density of correctly matched solvent-silica slurries.

Peak Wavelength and Absorbance Measurements

In practice the effects of background scattering can be minimized if peak wavelengths are located and absorbances are measured above a slant base line. The points of tangency with the apparent upper and lower wavelength limits of the peak were connected by a straight line. Peak wavelength was the wavelength of the point of tangency of the tangent to the curve which was parallel to this slant base line. Peak wavelengths, so defined, agreed well with those of the identical compounds in the absence of background scattering, whereas the apparent peak positions (above an assumed horizontal base line) are the sums of the background and compound absorbances, which always shift the apparent location of a smaller peak toward that of the larger peak upon which it is superimposed. Absorbance was determined as the area bounded by the curve above the slant base line, which was found to be directly proportional to the scalar absorbance measured between the slant base line and the determined peak parallel to the absorbance axis of the chart paper.

Extent of Elution by Slurrying Solvent

To test the extent of elution of relatively nonpolar compounds by the slurrying solvent during the course of the silica-UV spectrophotometry, the supernatant from a slurry coated with a monolayer containing 5 moles per cent tocopherol in stearic acid was examined. The slurry was prepared without any dry-mixing dilution of the coated silica. The supernatant showed less than 8% of the total tocopherol present in the system. Under the condition of 1:10 dry-mixing dilution, the supernatant contamination by elution is reduced to vanishingly low levels. Since the usual powder dilution is 1:10, the method gives reasonably quantitative absorbance values and

highly reproducible wavelengths of maximum absorption referable to compounds adsorbed in the monolayer rather than dissolved in the solution phase.

Silica-UV Spectrophotometry of Reference Compounds

To identify the absorption bands present in the silica-UV spectrum resulting from a mixture of reaction products, the wavelengths of maximum absorption and the molar absorptivity of authentic analogous reference compounds were determined on silica. This procedure permits tentative resolution of a mixed spectrum into its components. It also aids greatly in tentative classification of unknown compounds, since the extent of red and blue spectral shifts are very diagnostic. Large shifts also confirm that the moiety of a compound producing the shifted band is within the silanol electrostatic field, if not directly hydrogen-bonded, and thus confirm location and orientation in the monolayer.

Table I gives a list of relevant purified reference compounds whose spectral indices have been determined in solvent and on silica in this laboratory.

As discussed above in connection with tocopherol, red shifts appear characteristic of the π-π^* transition of quinones, unsaturated aldehydes and ketones; blue shifts appear to be characteristic of the similar transition in aromatic compounds with electron-donating substituents. Little or no shift occurs for polyenes without a conjugated donating or withdrawing group.

Our work has indicated that tocopherol and its derivatives with intact aromatic and chroman ring structure experience a blue shift in maximum and a decrease in molar absorptivity from the solvent values upon adsorption on silica (Fig. 1), whereas dienes or monoenes conjugated with a carbonyl group as in tocopheryl quinone (Fig. 2) or the dienone group of the dimer (Fig. 3) experience a red shift.

Thus, the tocopherol dimer, whose proposed structure (10,11) has separate aromatic and homoannular diene ketone moieties, not conjugatedwith each other, would be expected to have a moderate blue shift of the 3020 A band and a very strong red shift of the 3410 A band. A decrease in absorbance of the 3020 A band would be also expected. These changes were found (Fig. 3), giving further confirmation to the proposed structure, about which doubts have been recently expressed (12). The tocopherol trimer (8) is reported to contain two aromatic rings with associated chroman groups, together with an alpha-beta unsaturated cyclic ketone. The silica slurry spectrum (Fig. 4) showed the expected blue shift of the aromatic band at 2945 A and the red shift of the shorter wavelength absorption of the unsaturated carbonyl (appearing as end absorption below 2400 A in the solvent spectrum).

The small blue shift of the tocopheryl ethers in contrast to that of tocopherol and the chroman group of the dimer, appears to result from the anomalously short wavelength of their π-π^* band (2880 A in the solvent cyclohexane-cyclooctane, 8:2). The ethers are examples of molecules with the steric inhibition of resonance found in 2,6-dialkylated, alkyl-aryl ethers (13). Since donation of lone pair electrons to the ring is thus reduced, the wavelength of maximum absorption in solvent is abnormally short and bonding to silica produces less blue shift.

Unstrained six-membered ring formation involving the ether oxygen is known to remove these steric distortions. Therefore, in tocopherol and the aromatic band of its dimer, wavelengths in solvent are longer and blue shifts due to silica bonding are greater. The shorter wavelength of the aromatic band of the trimer in solvent in contrast to that of the dimer, and its smaller blue shift in silica slurry are consistent with greater steric distortion of the chroman rings than that in tocopherol and the dimer. The proposed molecular structure (8) would be expected to show steric strain.

Thus, for a given type of compound to be expected from a surface reaction, authentic analogue compounds may be studied in the adsorbed state on silica to determine the wavelengths of maximum absorption and the molar absorptivity. The amount of a known compound adsorbed may be calculated and the components of a spectrum resulting from two or three compounds present on the silica may be tentatively predicted and confirmed upon elution and separation. In addition, unknown compounds eluted from the silica surface and separated by appropriate methods may be classified by the extent and direction of the wavelength and absorptivity shifts of their spectra in solution as compared to those in silica slurry. This affords a valuable clue to the nature of the compound.

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UV Spectrophotometry of Autoxidized Lipid Monolayers While on Silica Gel

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ABSTRACT

Autoxidation reactions of linoleic acid and tocopherol and of mixtures of the two have been monitored in the dry monolayer state while adsorbed on silica gel, by means of silica slurry spectrophotometry and oxygen uptake measurements. The UV absorbance of the products of linoleic acid alone is similar to that of previously characterized compounds, but that from tocopherol is not referable to any oxidation products known to the authors, regardless of whether the determination is made while adsorbed on silica or on the extracted products. Two compounds, designated A and B, having absorbance in ethanol at 2610-2670 and 3000 A, respectively, are the major products of tocopherol oxidation detectable by UV above 2200 A. They are a tocopheryl quinone without a hydroxyl group and an adduct of linoleic acid and oxidized tocopherol. The product distribution suggests that tocopherol may have a restricted migration in this system.

INTRODUCTION

A method was developed in this laboratory for visible and UV spectrophotometry of lipid monolayers after adsorption on silica gel from solution in hydrocarbon solvents (1). The work reported here is the application of that method to study of the autoxidation of monolayers of linoleic acid and tocopherol, from which a new compound of autoxidized tocopherol and linoleic acid has been reported (2).

The compounds resulting from photodecompositions of molecules conducted on silica surfaces by Leermakers (3) suggest strongly that the migrational mobility of molecules which can act as hydrogen bond donors or acceptors is strongly restricted by the silica. Under these conditions, quantum yields are reduced by recombination of free radical intermediates and fewer product species are found. It was hoped that in the studies reported here on autoxidation of monolayers of compounds while on silica gel, a different and perhaps simpler product distribution might be found than that for autoxidations conducted in other, more conventional media. Finally, it was believed that the monolayer on silica provides a reasonable model system analogous to that of the lipoprotein membranes in freeze-dried foods, where lipids of the membranes are relatively dry and presumably firmly anchored to the protein (4) and the structure is paucilayered and porous. Autoxidation of these lipids is responsible for many of the flavor changes due to rancidity (5).

EXPERIMENTAL PROCEDURES

Materials

Silica Gel G was supplied by Warner-Chilcott Laboratories Instruments Division, Richmond, Calif., and the predecessor company, Research Specialties Co. The material has a particle size of 5-25 μ, and prior to acid washing, contains about 13% calcium sulfate as binder (6). The pore volume is reported by the supplier to be approximately 0.68 cc/g, median pore diameter, approximately 30 A, and specific surface area by nitrogen adsorption, approximately 450 m^2/g (K.P. Brinkmann, personal communication). Determinations at this laboratory by atomic absorption spectrophotometry

TABLE I

Adsorption of Linoleic Acid on Silica From Petroleum Ether Solution

Volume of petroleum ether, ml	Initial weight of linoleic acid, mg in solution	Weight of acid-washed silica, g	Weight of linoleic acid adsorbed[a], mg/2 g silica	Percentage adsorption, of initial weight
45	475	2.00	383	81
45	475	2.00	369	78
Mean			376	79

[a]Determined gravimetrically.

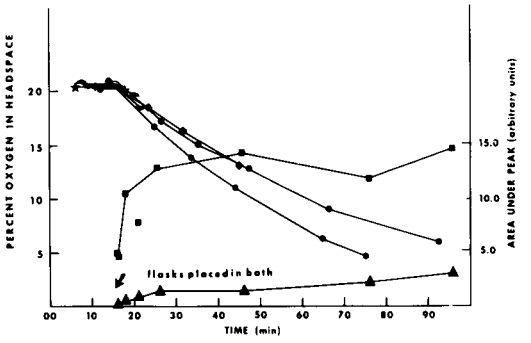

FIG. 1. Oxygen uptake and silica gel-UV spectrophotometry of autoxidation of linoleic acid monolayer at 80 C. Area of band centered at: ■ 2300 A; ▲ 2900 A.

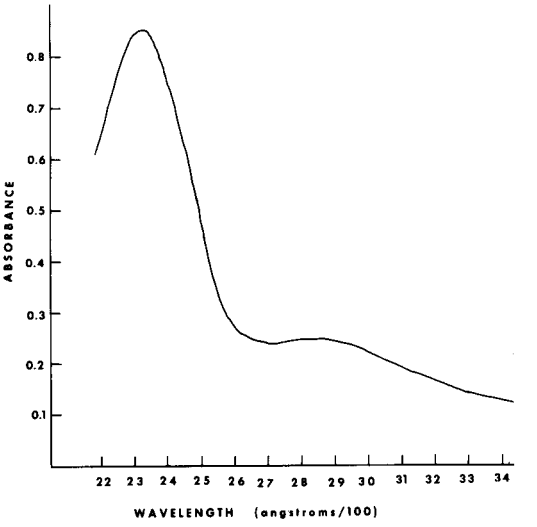

FIG. 2. UV absorption spectrum of autoxidation products of linoleic acid while in monolayer on silica. Sample taken after 80 min at 80 C.

of metal content removable by acid wash showed iron to be 162, copper 1.8, cobalt 2.8 and manganese 2.2 ppm. An acid wash procedure followed, which was essentially that of Stahl (7) with the exception that a mixture of cold, concentrated hydrochloric acid, concentrated nitric acid and water (1:1:2 v/v) was used. Five hundred grams of silica were stirred 10 min in a liter of this mixture, allowed 2 hr to settle and the supernatant liquid decanted. The procedure was repeated four times, and the silica was then washed four times in a Büchner funnel with a liter of deionized water or until the washings were neutral. A wash with 300 ml of ethanol and 200 ml of benzene and 24 hr oven-drying completed the procedure. Iron content of the final acid washings was reduced to 0.75 ppm, as determined by atomic absorption spectrophotometry. This procedure increased by threefold the antioxidant-dependent induction period of linoleic acid when in monolayer on silica.

Redistilled, deoxygenated, low-boiling (35-52 C) petroleum ether was used as solvent for deposition of compounds as monolayers from solution by adsorption on silica gel. All solutions were prepared in an atmosphere of dried and purified nitrogen procured from either Linde, Medical-Technical Gases, Inc., Medford, Mass., or Air Reduction Company, New York, N.Y.

Both synthetic *dl*-alpha-tocopherol (N.F. grade, Nutritional Biochemicals Corp., Cleveland, Ohio) and natural *d*-alpha-tocopherol (Distillation Products Industries, Rochester, N.Y., and Fisher Scientific Co., Fairlawn, New Jersey) were used. The synthetic material was used either as received (96% $C_{29}H_{50}O_2$) or after eight repetitions of an acid and alkali wash procedure (8). The natural material was used directly from the ampoule and gave a single spot in all systems of thin layer chromatography (TLC) and UV absorption was in agreement with literature values. Essentially identical results were obtained with tocopherol from all sources. Purification procedures are described elsewhere (2).

Linoleic acid was purchased from the Hormel Institute, Minneapolis, Minn. (stated purity 99%). Liquid partition chromatography on silicic acid with 2% methanol in benzene using a stationary phase of 20% methanol in benzene (9,10) gave a product not noticeably more free of compounds absorbing at 233 mμ than the original (less than 0.1% conjugated diene). Therefore, the original Hormel material was used in most of the experiments. It contains not more than 0.1 moles per cent of an impurity with the spectrum and chromatographic behavior of a conjugated trienoic acid.

IR spectrophotometry was carried out with a Perkin-Elmer IR Spectrophotometer, Model 21, using CCl_4 as solvent. A Cary Recording Spectrophotometer, Model 14 M, was used for UV spectra. Determination of molecular weight was by peak-matching mass spectrometry performed on a CEC Mass Spectrometer, Model 21-110B. Details are reported elsewhere (2). In photooxidation studies, there was employed an unfiltered Rayonet Super-kill Tube Type Germicidal Lamp using 110 v at 1.5 amperes, procured from Southern New England Ultraviolet Co., Middletown, Conn.

The diene ketone of linoleic acid, although not totally separated from linoleic acid, was characterized by a positive carbonyl test with

TABLE II

Oxygen Uptake and UV Absorbance in Silica-Cyclohexane Slurry of Linoleic Acid Autoxidation Products in Monolayer at 80 C

Time at 80 C, min	Headspace[a] oxygen, %	Wavelength[b] of maximum absorbance, A	Absorbance[c], arbitrary area units	Standard deviation, ±	Wavelength[b] of secondary maximum, A	Absorbance[c], arbitrary area units	Standard[d] deviation, ±
0	20.6 ± 0.21	2390	4.6	0.2	2900	0.18	0.05
0	20.6 ± 0.21	2390	5.1	0.4	2900	0.20	0.02
2	20.1	2390	10.4	2.4	2900	0.52	0.07
5	19.7	2375	7.8	0.6	2900	0.85	0.06
10	18.5	2380	12.9	2.7	2900	1.56	0.00
30	13.2	2380	14.3	0.8	2900	1.45	0.42
60	4.7	2370	11.9	1.5	2900	2.32	0.13
80	6.1	2360	14.7	1.5	2900	3.04	0.11

[a]Standard deviation shown for control samples was computed using standard formula with n-1, where n=6. Rapidity of oxidation precludes a statistical treatment in later samples.

[b]Computed using parallel to slant baseline.

[c]Computed above slant baseline.

[d]Computed using n-1, where n=3 silica-UV observations per sample.

2,4-dinitrophenylhydrazine, an IR spectrum showing peaks at 5.84, 6.03 and 6.14 μ (carboxyl, conjugated carbonyl C=O and C=C), and a UV absorption spectrum showing λ max 2780 A (ethanol), 2715 (cyclooctane-cyclohexane 2:8), 2870 (silica slurry in cyclooctane-cyclohexane 2:8), thus showing a strong red shift in polar solvents. The material having this UV absorption was extractible into dilute sodium carbonate solution and could be reextracted into ether on acidification.

Adsorption of Lipids for Autoxidation

Two gram portions of acid-washed silica were weighed, placed in 100 ml round bottom flasks and the silica activated by heating in a draft oven at 110 C for 1 hr. The flasks had been cleaned before use by soaking in a mixture of hot, concentrated nitric acid, hydrochloric acid and water (1:1:1 v/v), with thorough rinsing in tap water and deionized, distilled water. The flasks with the silica were outgassed under reduced pressure for at least 30 min, after which the vacuum was broken under dry nitrogen. Subsequent operations until the measurement of oxygen uptake were carried out in a dry box under dry nitrogen.

Measured volumes of petroleum ether solution containing varying molar ratios of lipid (usually 45 ml containing about 10 mg/ml of lipid) were added to the silica, the flasks placed on a shaker operating at five oscillations per sec with a 4 cm excursion, and shaken for 40 min. At the end of this period, the gel was allowed to settle 3 min and the clear, supernatant solution was decanted. Tests had shown that with nonpolar solvents, there is virtually no silica lost by this procedure. Initial solution concentration was adjusted so that at equilibrium adsorption conditions, the lipid concentration of the remaining solution would be that required to produce 75% of monolayer adsorption, according to previously prepared Langmuir adsorption plots. As a check on the amount adsorbed, silica gel spectrophotometry was conducted on known amounts of authentic compounds after virtually total adsorption on the gel (at low solute concentrations), the supernatant being essentially without UV absorption. Extinction coefficients thus obtained permitted confirmation of the results of gravimetric assays of equilibrium adsorbing solutions as to amounts adsorbed at a given concentration.

The flasks were placed on a dual manifold permitting solvent removal under a continuous stream of nitrogen at slightly above atmospheric pressure. An escape manifold permitted the monitoring of silica loss by blow off. This

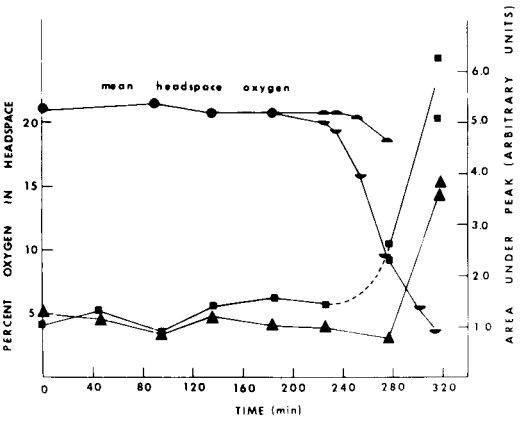

FIG. 3. Oxygen uptake and silica gel-UV spectrophotometry of autoxidation of linoleic acid monolayer containing 0.1 moles per cent tocopherol. Temperature 80 C. Shaded area includes means of two experiments. Area of band centered at: ■ 2300 A; ▲ 2900 A.

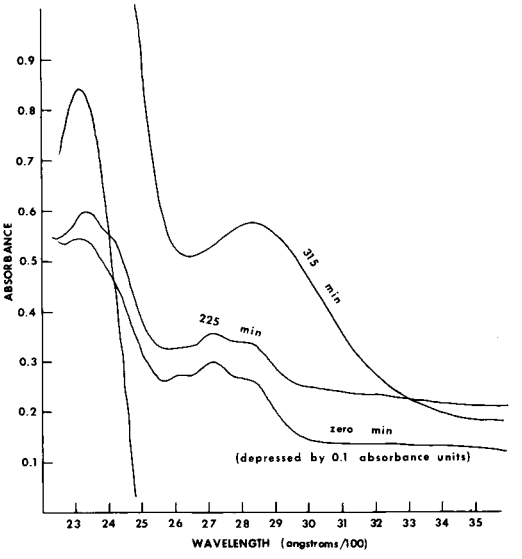

FIG. 4. UV absorption spectra on silica of products of autoxidation of linoleic acid containing 0.1 moles per cent tocopherol in monolayer on silica. Temperature 80 C.

was minimized by control of the nitrogen flow. Gas partition chromatography showed that solvent removal was complete after 1 hr. At the end of the solvent removal procedure, nitrogen flush was halted and the nitrogen line switched to a stream of laboratory air filtered through a Drierite column. After 10 min, flasks were tightly capped with a rubber serum bottle stopper (11).

Oxygen Uptake Determination

Progress of oxidation was followed by determining oxygen content of the headspace gas, using the gas chromatographic method developed by Bishov and Henick (11). The Fisher Gas Partitioner, Model 25 M, with a Sargent Recorder, Model SR, was used. Samples of 30-50 μl were withdrawn by a conventional gas chromatography Gas-Tight Syringe No. 1710, Hamilton Co., Inc., Whittier, Calif.

Flask gas volume, after correction for adsorbent volume, was 154 ml. At 25 C and standard pressure, 1.32 mmoles of oxygen were therefore contained in the enclosed air. The amount of lipid in the 45 ml of adsorbing solution, typically 475 mg or 10.6 mg/ml in the case of linoleic acid, was adjusted so that it constituted about 125% of a molecular equivalent of the oxygen available (371 mg of linoleic acid). Since the supernatant liquid (40 ml) was decanted after the adsorption period and prior to the solvent removal with nitrogen flush, and the concentration of the supernatant liquid was approximately 2.5 mg/ml, only 375 mg or about 75% of full theoretical Langmuir monolayer coverage, was deposited. Thus the effective charge was about a molecular equivalent of the oxygen available. This was done to study in detail only the oxygen uptake prior to and just after the end of the induction period. A low oxygen volume and a high sensitivity setting of the Gas Partitioner gave the desired sensitivity in oxygen uptake measurements. The rate-limiting effect of oxygen depletion on thin layer autoxidation has been shown to appear only at less than 1% headspace oxygen and was deemed not important in this study (12).

Before insertion of the flasks in the oil bath used to conduct the reactions, control readings of oxygen content were made on all flasks. Two flasks containing only a 2 g charge of dry silica were used as thermal and instrumental controls and as a correction for any oxygen uptake by the rubber stoppers. The difference between the oxygen content determined therein and 20.9% was used as a linear correction for other oxygen content measurements. Flasks were placed on an agitating rack in a thermostatically regulated oil bath held at 80 ± 2 C. Necks of the flasks protruded through holes in a "Styrofoam" blanket wrapped in aluminum foil, providing access for sampling. The rack was agitated at 4 cycles/sec with a 1 cm excursion.

Silica Slurry Spectrophotometry

UV spectra of the lipids, while adsorbed on silica, were obtained by methods reported elsewhere (1). At appropriate intervals, samples were withdrawn immediately after an oxygen uptake determination. Powder mixing dilutions

TABLE III

Relationship of Silica-UV Absorbance to Concentration for Reference Compounds (1)

One area Unit, Approximately	Absorbing on Silica at
0.08 Moles per cent[a] conjugated trienoic acid	2720 A
0.19 Moles per cent diene hydroperoxide[b] or alcohol	2370 A
0.26 Moles per cent alpha-beta unsaturated aldehyde	2270 A
0.48 Moles per cent alpha-beta unsaturated ketone	2295 A
0.17 Moles per cent diene aldehyde	2870 A
0.16 Moles per cent diene ketone	2870 A

[a]Relative to the linoleic acid content.

[b]Estimated by comparison with sorbyl alcohol.

ranged from 1/50 to 1/10, the latter being more common. Both peak wavelengths and absorbances were determined by graphic integration of area above a slant baseline as previously described (1).

Silicic Acid Column Chromatography

In an analysis of tocopherol oxidation products, the details of which are reported elsewhere (2), silicic acid column chromatography in benzene followed by gel filtration in chloroform was used. The silica containing the autoxidized products in monolayer form was dusted into a thin layer of solvent lying above a filter paper disk placed above the chromatographic silica slurried in solvent. A second disk was placed above the lipid-coated silica and elution commenced. No anomalies of separation were experienced due to this procedure, and weights of products recovered after scouring with methanol were between 75% and 95% of the total linoleic acid and tocopherol initially in the sample. After evaporation of chromatography solvent under reduced pressure and nitrogen flush and determination of weight, fractions were dissolved in 10 ml 95% ethanol. The accumulated UV absorption area above 2500 A recoverable from the column was 89% to 91% of the area found in silica-UV studies after correction by the appropriate dilution and molecular extinction factors. The form and area of the silica-UV curve can be reproduced by appropriate addition of the spectra of the chromatographed components as shifted on silica.

Characteristics of Tocopherol Derivatives Eluted From Oxidized Linoleic Acid-Tocopherol Monolayer

Compound A. Yellow oil; molecular weight (mass spectrometry) 428.4; R_f (TLC on silica G with benzene) 0.57, α-tocopherol 0.32, α-tocopheryl quinone 0.04; IR (μ) very strong carbonyl at 6.07, no peak in the 2.8 to 3.0 region, but the balance of the spectrum resembles that of α-tocopheryl quinone; with $SnCl_2$-HCl treatment UV maximum is shifted to 2930 A; extracted product has the R_f of tocopherol and gives positive Emmerie-Engel test; unreduced compound gives negative test.

Compound B. Nearly colorless oil; molecular weight (mass spectrometry) 708.6; R_f (TLC on silica G with benzene) 0.08, linoleic acid 0.07; IR (μ) linoleic acid peaks plus strong peaks at 7.24 and 9.12 (methyls and chroman of tocopherol or congeners); with BCl_3-MeOH treatment gives a relatively nonpolar compound with R_f of 0.42 in silica G-benzene TLC; methyl ester of linoleic acid has R_f of 0.40 in this system; neither acid nor ester of Compound B gives positive Emmerie-Engel test.

RESULTS AND DISCUSSION

Autoxidation of Linoleic Acid on Silica

Linoleic acid was chosen as representative for silica-UV spectrophotometry of the products of autoxidation of a methylene-interrupted diene system typical of biological membrane lipids. The acid was used rather than the triglyceride or methyl ester because of the high binding energy of the carboxyl group to the silanol surface. It was thought that this might restrict the mobility and give a known orientation of the acid in a solvent free monolayer during autoxidation. It would also reduce elution by the slurrying solvent in silica-UV procedures. The acid is available in relatively pure form, the only spectral interfering contaminant being less than 0.1 moles per cent of a material whose spectral and chromatographic behavior conform to that of conjugated trienoic acid. This is quickly destroyed in rapid phase oxidation. Typical adsorption data are shown in Table I.

The mean value of 188 mg/g of uncoated silica represents about 75% of the theoretical saturation monolayer weight ratio at infinite equilibrium concentration derived from a Langmuir plot of adsorption data for linoleic acid. Final equilibrium concentration is usually about 8×10^{-3}M. About 11 mg of linoleic acid are deposited from the remaining adherent solution by solvent removal under nitrogen after decantation of 40 ml of the equilibrium

solution. It can be assumed that due to the non-equilibrium deposition, part of this 3% of the coating may be nonmonolayer in nature, but this seems insufficient to cause concern.

Six flasks containing the above charge, after solvent removal and replacement of an air atmosphere, were shaken at 80 ± 2 C for periods up to 100 min. At appropriate intervals, the headspace gas was sampled and a flask withdrawn for silica-UV study. The spectral absorbance data (areas between the slant baseline and the curve) were expressed in arbitrary areal units. Table II and Figures 1 and 2 give the results.

Figure 1 shows a rapid, immediate, nearly linear uptake of oxygen, a corresponding rapid increase in absorbance in the 23-2400 A region within the first 5 min and a slower increase of absorbance in the 2900 A region. Table III shows the approximate conversions of absorbance area to concentration, for possible compounds absorbing in these regions, the values being relative to the initial linoleic acid charge.

Because of the similarity of peak wavelengths on silica of compounds absorbing at or near 2300 A (diene peroxides or alcohols, alpha-beta aldehydes and ketones), the method cannot distinguish between species in this region. However, it can be said that the absorption in this region rises very rapidly in the first 10 min and approximately triples during the entire 90 min of the rapid autoxidation of linoleic acid without antioxidants. The final value is consistent with either about 3 moles per cent conjugated diene hydroperoxide or diene alcohol, 4 moles per cent alpha-beta aldehyde or 7 moles per cent alpha-beta ketone, or appropriate mixtures of these compounds.

In the 2800-2900 A region, there is little ambiguity, since separate carbonyl tests, silica-UV spectral red shift, ether extractability in acid vs. base, IR spectrum and chromatographic behavior of the eluted products show that the major compound absorbing here is the conjugated diene ketone derivative of linoleic acid. The final value corresponds to about 0.5 moles per cent. The conjugated trienoic acid contaminant is autoxidized quickly and disappears from this region. It is plain that diene carbonyl is present very early in the rapid autoxidation on silica of linoleic acid without antioxidants.

These spectral absorbances are substantially below those usually found in bulk oil oxidations. They are also much lower than values suggested by peroxide or carbonyl tests performed on the compounds while on the silica, suggesting that the latter values may be partly test artifacts.

TABLE IV

Oxygen Uptake and UV Absorbance in Silica-Cyclohexane-Cyclooctane Slurry of Linoleic Acid Monolayer Containing 0.1 Moles Per Cent Tocopherol, 80 C

Time at 80 C, min	Headspace Oxygen, %	Standard deviation[a], ±	Wavelength[b] of maximum absorbance, A	Absorbance[c], arbitrary area units	Average error[d], ±	Wavelength[b] of secondary maximum, A	Absorbance[c], arbitrary area units	Average error[d], ±
0	20.9	0.11	2360	1.05	0.12	2725	1.22	0.03
45	---	---	2360	1.34	0.15	2720	1.16	0.08
92	21.2	0.18	2360	0.91	0.28	2730	0.87	0.12
135	20.6	---	2390	1.39	0.04	2725	1.21	0.08
183	20.8	0.08	2370	1.55	0.10	2725	1.03	0.03
225	20.6	0.16	2370	1.45	---	2730	0.96	---
276	18.6	---	2370	2.46	0.17	2830	0.80	0.01
315	3.7	---	2360	5.66	0.57	2870	3.66	0.12

[a]Computed using standard formula with n-1, where n varied from 6 to 4.
[b]Computed using parallel to slant baseline.
[c]Computed above slant baseline.
[d]Computed as $\pm 1/n\ (\sum d)$, where $n \geqslant 2$ and d=absolute value of $(x-\bar{x})$.

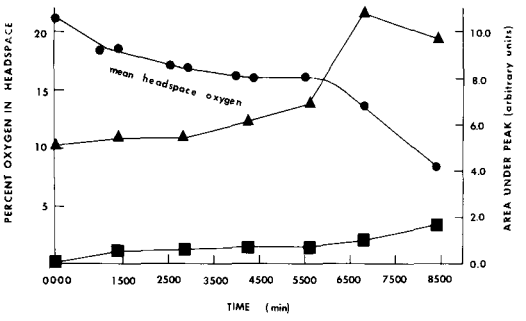

FIG. 5. Oxygen uptake and silica gel-UV spectrophotometry of autoxidation of linoleic acid monolayer containing 4.3 moles per cent tocopherol. Temperature 80 C. Shaded area includes means of four experiments. Area of band centered at: ■ 2300 A; ▲ 2800 A.

Autoxidation of Linoleic Acid on Silica as Modified by 0.1 Moles Per Cent Tocopherol

The effect of tocopherol on linoleic acid autoxidation was studied by silica-UV methods. One tenth mole per cent tocopherol was added under nitrogen to the absorbing solution prior to the addition of linoleic acid. Oxidation conditions, monitoring of headspace gas, sampling, silica-UV readings and data analysis were performed as above, with the exception that the period of study was lengthened to 4-5 hr, because the tocopherol produced a 240 min period with essentially no oxygen uptake at 80 C within the detection limits of the system. It was found that a cyclohexane-cyclooctane ratio of 4:1 gave best results in the spectrophotometry of the mixed monolayer. Table IV and Figures 3 and 4 display the results.

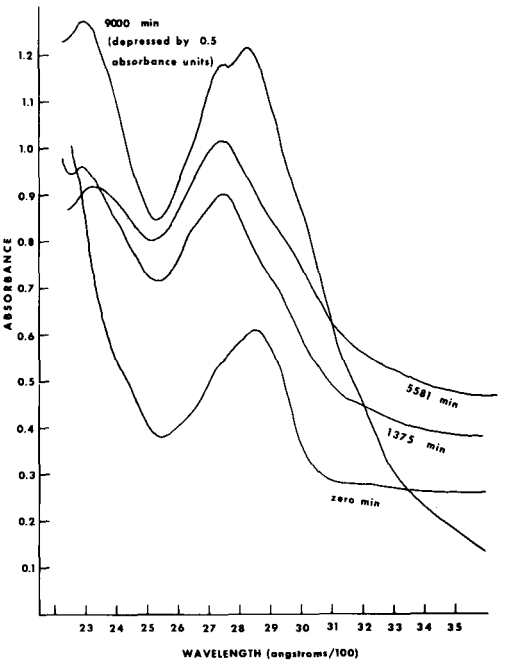

FIG. 6. UV absorption spectra on silica of products of tocopherol oxidation mediated by linoleic acid in monolayer on silica. 4.3 Moles per cent tocopherol. Temperature 80 C.

The same conversions of area of UV absorbance to concentration apply as was the case above when no tocopherol was present, since spectral absorbance due to tocopherol or its products was too low for detection.

Again, although the 2300 A region is anomalous, it can be stated that if the absorp-

TABLE V

Wavelength Maximum and Absorbance of Alpha-Tocopherol in Various Media

Medium	Wavelength Maximum, A	Absorbance, $E^{1\%}_{1cm}$	Source
95% Ethanol	2920	73.7	(13)
Glacial acetic acid	2915	69.0	This laboratory
Cyclohexane-cyclooctane (8:2)	2980	87.5	This laboratory
Precoated silica slurried in cyclooctane-cyclohexane (7:3)	2880[a]	52.3[a] ± 3.0[b]	This laboratory
Silica added to tocopherol in cyclooctane-cyclohexane (2:8)	2880[a]	56.8[a] ± 2.0[c]	This laboratory

[a]Slant baseline used. See text.
[b]Mean ± standard deviation, n=4.
[c]n=2.

tion is all due to diene hydroperoxide, as is probably true in the initial stages, the amount rises from 0.2 to 0.3 moles per cent during the approximately 240 min of the induction period. It then rises very rapidly to an amount of 1.2 moles per cent for the last observation, when 82% of the available oxygen has been consumed. In this case, probably because the linoleic acid was placed directly into a tocopherol-containing medium, the initial and final values in the 2300 A region are less than one third of those in the experiment without tocopherol.

In the 2700-2900 A region, the initial absorption can be traced to the contaminant of about 0.1 moles per cent conjugated trienoic acid, whose characteristic three peak absorption is initially visible in Figure 4. The 0.1 moles per cent tocopherol would correspond to only 0.07 area units of silica-UV absorption, or about 6% of the trienoic acid absorption. This amount would not be separable from the background level of the instrument, and cannot be responsible for the observed slow decline of UV absorption in this region throughout the induction period.

At the end of the induction period, rapid absorption increases are observed in the 2700-2900 A region and the peak wavelength shifts to 2870 A, which can probably be assigned to increase in diene carbonyl, since tocopherol autoxidation products absorbing in this region do not give substantial increase in UV absorption at this wavelength compared to that of the original tocopherol (1). The trienoic acid absorption disappears slowly in this area during the induction period.

The comparatively small increase in absorption in the 2300 A region, during the induction period, corresponding at maximum to only 0.1 moles per cent of diene, if this is its origin, suggests that, if conjugated diene hydroperoxide is the chain propagator in a relatively anchored array of linoleic acid and tocopherol molecules, it rapidly forms secondary products, largely without UV absorption.

Autoxidation of Tocopherol Mediated by Linoleic Acid

The presumed molecular anchoring, dehydrated, porous structure and monolayer configuration of a mixture of linoleic acid and tocopherol on silica appear to provide a plausible model for freeze dried biological membrane systems in respect to autoxidation, since the protein sheet to which lipid constituents are presumed to be affixed (4) may have polarity and hydrogen bonding characteristics somewhat analogous to the silica surface.

TABLE VI

Oxygen Uptake and UV Absorbance in Silica-Cyclohexane-Cyclooctane Slurry of Linoleic Acid Monolayer Containing 4.3 Moles Per Cent Tocopherol, 80 C

Time at 80 C, min	Day	Headspace oxygen, %	Standard[a] deviation, ±	Wavelength[b] of maximum absorbance, A	Absorbance[c], arbitrary area units	Averaged[d] error, ±	Wavelength[b] secondary maximum, A	Absorbance[c], arbitrary area units	Averaged[d] error, ±
0	0	21.1	0.15	2850	5.12	0.32	None	None	--
1375	1	18.8	0.27	2770	5.46	0.06	2310	0.58	0.00
2819	2	17.5	0.39	2760	5.43	0.27	2340	0.63	0.17
4247	3	16.3	0.51	2760	6.20	0.18	2350	0.77	0.06
5581	4	16.0	0.32	2750	6.90	0.33	2350	0.74	0.03
6692	5	15.3	1.28	2760	10.74	0.47	2360	1.01	0.23
8057	6	9.2	2.37	2825	9.66	0.17	2350	1.74	0.09

[a]Computed using standard formula with n-1, where n varied from 9 to 4.
[b]Computed using parallel to slant baseline.
[c]Computed above slant baseline.
[d]Average error was computed as $\pm 1/n\,(\sum d)$, where n=2 and d=absolute value of $(x-\bar{x})$.

TABLE VII

Spectral Shifts of Oxidized Products Eluted From Linoleic Acid-Tocopherol Monolayer

Compound	Wavelength of maximum absorption, A		Spectral shift, cm^{-1}
	Cyclohexane-cyclooctane solution (8:2 v/v)	Silica gel-cyclohexane-cyclooctane slurry (8:2 v/v)	
Compound A (quinone)	2595-2675	2700-2750	1020 (red)
Compound B (adduct of tocopherol and linoleic acid)	3010	2930	907 (blue)
Diene ketone of linoleic acid	2715	2870	1989 (red)

Therefore, a study was planned, using 5 moles per cent tocopherol included in a linoleic acid adsorbing solution, to survey the products of tocopherol oxidation by silica-UV methods. The actual tocopherol amount adsorbed, as determined from silica-UV measurements, was approximately 4.3 moles per cent. Experimental procedures were similar to those above, but the time of study was seven days at 80 ± 5 C.

Silica gel-UV spectrophotometry on preparations of tocopherol adsorbed alone or with stearic acid on silica shows that the wavelength of maximum absorption is shifted to 2880 A from its value of 2920 A in ethanol and 2980 A in cyclohexane. In addition, the extinction coefficient derived from absorbance above a slant baseline (1) varies from 71% to 77% of its value similarly measured in ethanol solution. Representative values of E are shown in Table V.

Table VI and Figures 5 and 6 show the results of autoxidation of tocopherol present as 4.3 moles per cent of a monolayer containing 185 mg of linoleic acid per gram of uncoated silica.

Clearly shown in Table VI and Figure 6 is the abrupt shift of the peak of absorption from 2850 to 2770 A within the first day of a four-day induction period. This coincides with a pronounced consumption of oxygen which then levels off. However, in the first day, approximately 10% of available flask oxygen has been consumed, and by the end of the induction period, as shown in Figure 5, nearly 25% of the available oxygen has been consumed. That this is still a tocopherol dependent induction period of sorts, however, is shown by the sharp increase in oxygen consumption after four days (5581 min sample).

The end of the induction period is marked by a rather abrupt shift of the peak of absorbance in silica-UV back to 2830 A. This coincides with a rapid increase in diene ketone as revealed by chromatographic product analysis.

The area under the major absorbance peak as shown in Figure 6 and Table VI increases relatively slowly and the curve remains essentially similar until the end of the fourth day, the end of the induction period. It then sharply rises and changes form, leveling off and finally falling in the rapid phase of oxidation, days five and six.

The area under the curve of the secondary maximum at 2300-2400 A is nonexistent in the first sample, but approximates at one day an amount that could be produced by 0.1 moles per cent conjugated diene hydroperoxide. It increases to represent 0.15 moles per cent at the end of the induction period, and then rises much more sharply to slightly over 0.3 moles per cent. It should be cautioned as before that this area may correspond as well to differing amounts of alpha-beta aldehyde or ketone or to conjugated diene alcohol. In any case, it is remarkable for its small amount considering the fact that the oxygen uptake is so large and the amount of tocopherol destroyed is so great. As mentioned above, at this temperature, conjugated diene hydroperoxide may be rapidly converted to nonconjugated materials.

The silica-UV spectrophotometry gave no evidence for known characterized monomers, dimers or trimers reported from tocopherol oxidation (14-27). The dimers and trimers known have very characteristic absorptions on silica (1), particularly in the 3700 and 2400-2500 A region, respectively, which would have certainly been detected at the tocopherol loading used,

either on the silica or in subsequent total chromatography. Characteristic UV bands exist for all other known monomers, such as the tocopherones (27), and these were not seen. Although Compound A (below) is a quinone, its UV spectrum in either solvent or slurry differs from that of authentic α-tocopheryl quinone.

The results of silicic acid chromatography of the products of autoxidation from four and five day samples (at the onset of rapid phase oxidation) showed that two products derived from tocopherol very strongly predominated. The first, a relatively nonpolar compound designated "Compound A", appears as the first important product eluted after about one column volume of benzene eluant. It is derived from about 10% of the available tocopherol. The second, designated "Compound B", appears after four or five column volumes and represents 40% of the available tocopherol.

The spectral shifts of the two products and of the linoleic acid diene ketone obtained as a minor product after tocopherol depletion are shown in Table VII. Silica slurry spectrophotometry facilitates preliminary classification of such unknown compounds. Thus, Compound A shows a double peak and a moderate red shift, Compound B a moderate blue shift and the diene ketone a strong red shift. Spectral shifts of analogous reference compounds (1) are consistent with the tentative assignment of Compound A as a quinone, Compound B as a tocopherol derivative with an intact aromatic ring, and the ketone as a multiply unsaturated carbonyl.

These tentative assignments are confirmed by the additional chemical and spectral data, some of which has been reported above under Experimental Procedures for the two tocopherol derivatives. Details of chromatography and the characterization of Compound B are reported elsewhere (2).

Neither of these materials show characteristics which coincide with any tocopherol oxidation product known to us. Compound A is a quinone, but it has no hydroxyl group and its mobility in TLC is five times that of any known quinone of tocopherol. It produces the absorption in the silica-UV spectrum at 2760 A. Compound B is an adduct of linoleic acid and bivalently oxidized tocopherol. It produces the inflection in the silica-UV spectrum at 2930 A.

These two compounds at their maximum amounts represent about 50% of the available tocopherol on a molar basis. The remaining material appears not to be detectable by UV or chromatographic methods and may be polymerized.

One may conclude from these data that the oxidation of tocopherol coadsorbed with fatty acids in monolayer on silica gel, presumably mediated by linoleic acid hydroperoxide, results in products not previously found in other systems. It would follow that the course of the reaction in this matrix is different. Also, the failure to find dimers or trimers of tocopherol is consistent with a restricted migration of tocopherol.

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Diet and the Fatty Acids in the Plasma of Lambs During the First Eight Days After Birth

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ABSTRACT

This study reports the plasma lipid changes in lambs receiving either ewes' milk or a reconstituted low fat milk powder during the first eight days after birth. The liveweight gains of the lambs on the artificial diet was less than half that observed in the lambs on the natural diet. The plasma lipid levels in the lambs on the natural diet increased considerably after birth due to large increases in the concentrations of the cholesteryl ester, triglyceride and phospholipid fractions. For the lambs on the artificial diet these fractions remained similar to those observed at birth. In both groups unesterified fatty acid fractions decreased after birth. The main difference between the two groups of lambs was the large increase in C_{18} polyunsaturated fatty acids observed after birth in the lambs on the natural diet. The triene-tetraene ratio of the fatty acids in the lambs on the natural diet decreased from 1.0 at birth to 0.08 after eight days whereas in the lambs on the artificial diet the ratio increased to 2.9. These composition changes are discussed in relationship to the metabolism of essential fatty acids in monogastric animals.

INTRODUCTION

The fatty acid composition of tissues of the newborn animal depends upon the balance between those derived from maternal circulation and those synthesized by the fetus. It has been shown in several species (1-3) that little, if any, esterified fatty acids of the maternal circulation are taken up by the fetus during development. However, work with isotopically labeled palmitic acid (2,4) has demonstrated that unesterified fatty acids of maternal plasma may constitute an important source of fatty acids to the fetus. Although ruminant plasma contains high concentrations of linoleic and linolenic acids, these fatty acids are confined mainly to cholesteryl ester and phospholipid fractions (5). This specific distribution of polyunsaturated fatty acids between lipid fractions in the maternal blood might explain the low concentrations of essential fatty acids found in ruminant plasma at birth. In the ruminant the net placental flow of maternal fatty acids to the fetus has been shown to be low and it has therefore been suggested that in the ruminant fetus there is a greater dependence upon de novo synthesis of fatty acids (6). Although the concentration of linoleic and linolenic acids in the plasma of the ruminant at birth is very low, it has been demonstrated with lambs that concentrations of these polyunsaturated fatty acids undergo a considerable increase within the first 48 hr after birth (7). During this period the diet consists entirely of the ewes' milk and it has therefore been suggested that one of the functions of ewes' colostrum is to provide the newborn lamb with these essential fatty acids (7). However, investigations on the fatty acid composition of ewes' colostrum and milk during early lactation (8) have shown that the concentration of linoleic acid never exceeded 1% of the total fatty acids present, whereas the concen-

TABLE I

Total Fat and Linoleic Acid Content of Ewe Milk and Reconstituted Milk Powder

	Ewe Milk				
		Days	Post-partum[a]		
	Colostrum	1	2	3-8	Reconstituted milk powder
Total fat[b]	17.9	14.3	10.4	10.6	0.03
Linoleic acid[c]	0.64	0.68	0.86	0.77	1.20

[a]See Reference 8.
[b]Percentage by weight.
[c]Percentage of total fatty acids.

TABLE II

Mean Daily Live Weight Gains of the Lambs Receiving the Natural Diet and the Artificial Low Fat Diet

Days after birth	Natural diet, g/day	Low fat diet, g/day	S.E.
0 - 2	277	37	±39.5
2 - 4	205	72	±25.8
4 - 6	225	142	±59.4
6 - 8	294	183	±32.4

tration of linolenic acid was even lower. In an attempt to answer some questions concerning the metabolism of the C_{18} polyunsaturated fatty acids in the lamb during the first week after birth an experiment was conducted in which detailed lipid analysis was carried out on the plasma of lambs suckled naturally for eight days after birth and from lambs given an artificial low fat diet for eight days after birth.

EXPERIMENTAL PROCEDURES

Twin lambs were obtained from a flock of pure bred Cheviot ewes. The ewes had been given a diet of good hay and concentrates; water was given ad lib. The lambs were divided at birth into two groups. One member of each twin pair was allowed to suckle and stay with its mother while the other member was segregated from its mother and received a diet of a reconstituted low fat dried milk powder (Unigate Dairies Ltd., London). Food was allowed ad lib. Samples of the ewes' milk were obtained for analysis on the day of parturition and on each of the following eight days after parturition (8). The low fat diet was reconstituted with water to give a total dry matter content of 27% on the day of birth; the proportion of water was gradually increased so that on the eighth day after birth the lambs received the reconstituted diet containing only 11% dry matter. All the lambs received an intramuscular injection of a multivitamin preparation (Crooks Laboratories Ltd., London) on the second day after birth.

Blood samples were obtained from the jugular vein of each lamb immediately after birth before access to food; further blood samples were taken at exact intervals of one, two, three, four, six and eight days after birth. The lipids were extracted from the plasma by the method of Nelson and Freeman (9). The plasma lipids were fractionated into cholesteryl esters, triglycerides, unesterified fatty acids and phospholipids by thin layer chromatography and the fatty acid compositions of these lipid fractions were determined by the gas chromatographic methods described in more detail by Moore and Williams (10), Noble and Moore (11), and Moore et al. (12). Absolute concentrations of various lipid fractions in the plasma were determined by addition to each fraction of a known amount of *n*-heptadecanoic acid as an internal standard. No attempt was made to determine the concentration of free cholesterol in the plasma. The results were analyzed statistically on a paired treatment basis according to methods described in detail by Cochran and Cox (13).

RESULTS

Although the concentration of linoleic acid in milk fats was of the same order in both natural and artificial diets, concentration of total fat in the artificial diet was negligible when compared to that of the ewes' milk (Table I). Lambs receiving the natural diet maintained a consistently high growth rate as compared to lambs on the artificial diet (Table II).

Concentration of the total plasma fatty acid and concentrations of fatty acids in each of the lipid fractions of plasma from the naturally-fed and artificially-fed lambs after birth, are given in Table III. In contrast to the naturally-fed group, the concentration of the total plasma fatty acids in lambs receiving the artificial diet decreased immediately after birth and throughout the experimental period remained lower than that observed at birth (Table III). Of the four main lipid fractions of the plasma, the major contribution to the rise in total fatty acids observed in naturally-fed lambs after birth was provided by increases in concentrations of triglyceride and phospholipid fractions, with a smaller but significant contribution being provided by the cholesteryl ester fraction. The concentration of unesterified fatty acids of both groups of lambs on the other hand decreased significantly after birth.

The two main fatty acids of plasma triglycerides at birth were palmitic and oleic acids which together accounted for some 67% of the total fatty acids present (Table IV). Oleic acid in plasma triglycerides of naturally-fed lambs increased considerably after birth and this was

TABLE III

Concentration of Total Plasma Fatty Acids and Concentration of the Fatty Acids Circulating in Each of the Lipid Fractions of the Plasma From Lambs Receiving the Natural Diet or the Artificial Low Fat Diet[a]

Days after birth	Triglyceride fatty acids			Unesterified fatty acid			Phospholipid fatty acids			Cholesteryl ester fatty acids			Total fatty acid		
	Natural	LS[b]	Low fat	Natural	LS	Low fat	Natural	LS	Low fat	Natural	LS	Low fat	Natural	LS	Low fat
0	9.45		8.23	28.1		32.7	27.4		27.2	16.7		18.0	81.7		86.0
1	83.65[c]	$P<0.01$	8.25	21.5	$P<0.01$	8.83	100.2[c]	$P<0.01$	27.4	27.5[e]	$P<0.01$	12.9	232.8[c]	$P<0.01$	57.5
2	76.2[c]	$P<0.01$	7.30	16.0[d]		7.98[c]	122.1[c]	$P<0.01$	19.7	40.1[c]	$P<0.001$	14.3	254.3[c]	$P<0.001$	49.2
3	80.2[c]	$P<0.01$	6.48	12.2[c]		5.70[c]	96.0[c]	$P<0.01$	17.5	39.5[c]	$P<0.001$	9.2	227.9[c]	$P<0.001$	38.9
4	98.4[c]	$P<0.01$	7.23	16.2[c]	$P<0.05$	5.95[c]	111.7[c]	$P<0.01$	20.7	42.8[c]	$P<0.001$	12.1	269.1[c]	$P<0.001$	46.0
6	66.2[c]	$P<0.01$	6.35	16.6[d]	$P<0.01$	4.00[c]	92.2[c]	$P<0.01$	23.3	43.4[c]	$P<0.001$	9.6	218.4[c]	$P<0.01$	43.2
8	35.9		8.83	16.6[d]	$P<0.01$	5.40[c]	83.0[c]	$P<0.01$	25.8	39.8[c]	$P<0.001$	14.8	175.4[c]	$P<0.01$	54.7
SE within treatment between days		±15.58			±4.686			±12.14			±5.58			±25.85	
SE between		±16.27			±4.686			±15.03			±5.58			±28.92	

[a]Milligrams per 100 ml plasma.

[b]LS, level of significance of the difference between the value for natural and low fat diet.

[c]The difference between this value and the corresponding value for day 0 is $P < 0.001$.

[d]The difference between this value and the corresponding value for day 0 is $P < 0.01$.

[e]The difference between this value and the corresponding value for day 0 is $P < 0.05$.

accompanied by significant, although smaller, increases in stearic and linoleic acids. In the artificially-fed lambs the changes were less pronounced and were confined to an increase in stearic acid and a decrease in palmitoleic acid. However, palmitoleic acid in artificially-fed lambs remained significantly higher than that in lambs on the natural diet.

Oleic acid in plasma unesterified fatty acids, however, decreased after birth in both naturally-fed and artificially-fed lambs (Table V). Both groups also showed increases in palmitic and stearic acids. In artificially-fed lambs there was also an increase in palmitoleic acid, whereas in naturally-fed lambs it decreased as did also linoleic acid.

The C_{20} and C_{22} polyunsaturated fatty acids accounted for some 18% of the total fatty acids in plasma phospholipids at birth (Table VI) in spite of the very low concentrations of linoleic and linolenic acids. However, linoleic and linolenic acids increased rapidly after birth in naturally-fed lambs but remained very low in lambs on the artificial diet. The increase in C_{18} polyunsaturated fatty acids in naturally-fed lambs was accompanied by a rapid decrease in $\Delta^{5,8,11}$ eicosatrienoic acid. The eicosatrienoic acid-arachidonic acid ratio (triene-tetraene ratio) at birth was about 1.0 but after eight days on the natural diet the ratio decreased to 0.08. This ratio was 2.9 for lambs on the artificial diet after eight days.

During the first week increases in concentration of linoleic and linolenic acids in plasma cholesteryl esters (Table VII) of naturally-fed lambs were considerably greater than increases that occurred in concentrations of these acids in the phospholipids. In the artificially-fed lambs linoleic and linolenic acids remained low throughout the experimental period. As in phospholipids, eicosatrienoic acid in plasma cholesteryl esters of naturally-fed lambs remained similar to that observed at birth. Arachidonic acid in plasma cholesteryl esters of both groups of lambs decreased over the experimental period although this acid in the cholesteryl esters of naturally-fed lambs remained significantly higher than in artificially-fed lambs.

DISCUSSION

The plasma of the newborn lamb contains a large concentration of unesterified fatty acids and although it has been shown that during pregnancy the concentration of the unesterified fatty acid fraction in the plasma of the ewe is increased to a level well above that found in the nonpregnant sheep (4) it is now known that transfer from mother to fetus in sheep is

TABLE IV

Fatty Acid Composition of the Plasma Triglycerides From the Lambs Receiving the Natural Diet and the Artificial Low Fat Diet[a]

Days after birth	16:0			16:1			18:0			18:1			18:2		
	Natural	LS	Low fat	Natural	LS	Low fat	Natural	LS	Low fat	Natural	LS	Low fat	Natural	LS	Low fat
0	37.6		39.1	10.17		8.6	10.6		11.0	30.5		30.9	1.38		1.38
1	30.1[c]	$P<0.001$	39.3	2.73[c]	$P<0.01$	5.73[c]	11.0		16.7[e]	52.9[c]	$P<0.01$	26.1	1.85		2.08
2	32.2[d]	$P<0.001$	39.1	2.70[c]	$P<0.001$	7.63	12.6		16.5[e]	48.6[c]	$P<0.01$	25.5	1.95		2.08
3	27.7[c]	$P<0.001$	40.0	2.32[c]	$P<0.001$	6.37[c]	15.7		17.8[e]	49.3[c]	$P<0.01$	30.1	2.12		1.44
4	28.2[c]	$P<0.001$	35.5	2.45[c]	$P<0.001$	6.53[c]	16.8[e]		16.1[e]	49.7[c]	$P<0.01$	32.5	1.98		2.18
6	28.9[c]	$P<0.001$	36.2	2.48[c]	$P<0.001$	6.85	17.6[e]		14.0	46.0[c]	$P<0.05$	34.9	2.22[e]		2.04
8	32.4[c]	$P<0.05$	37.1	2.83[c]	$P<0.001$	7.75	16.3[e]		12.9	44.0[c]		37.4[e]	2.88[c]	$P<0.05$	1.65
SE within treatments between days		±2.07			±0.735			±2.73			±3.03			±0.417	
SE between treatments within days		±2.07			±0.752			±3.68			±4.04			±0.459	

[a]Major components, weight percentage of total. For statistical treatment see footnote to Table III.

TABLE V

Composition of the Plasma Unesterified Fatty Acids From the Lambs Receiving the Natural Diet and the Artificial Low Fat Diet[a]

Days after birth	16:0			16:1			18:0			18:1			18:2		
	Natural	LS	Low fat	Natural	LS	Low fat	Natural	LS	Low fat	Natural	LS	Low fat	Natural	LS	Low fat
0	24.9		24.7	4.2		3.8	15.6		16.9	50.5		48.5	1.1		2.1
1	27.9		32.0[e]	3.7	P<0.01	5.4[d]	13.7		17.1	47.4	P<0.01	30.4[c]	3.2		4.5
2	33.9[d]		35.1[c]	3.6	P<0.001	6.6[c]	17.9		20.6	41.4[e]	P<0.01	22.8[c]	2.8		2.6
3	28.8		33.5[d]	3.5	P<0.01	5.4[d]	20.0[e]		23.9[c]	40.0[e]	P<0.05	29.2[c]	3.9		4.1
4	33.5[d]		37.7[c]	2.9[e]	P<0.01	4.6	23.9[c]		18.9	34.5[d]		33.5[c]	3.2		1.4
6	30.7	P<0.001	41.6[c]	3.0[e]	P<0.001	6.0[c]	23.6[c]		19.6	36.3[d]	P<0.05	25.0[c]	5.3[e]		3.2
8	29.1	P<0.01	38.1[c]	2.9[e]	P<0.001	5.1[e]	22.7[d]		19.2	37.5[d]		31.9[c]	5.6[e]	P<0.05	1.3
SE within treatments between days		±3.00			±0.55			±2.18			±3.94			±1.81	
SE between treatments within days		±3.00			±0.56			±2.56			±4.07			±1.81	

[a]Major Components, weight percentages of total. For statistical treatment see footnote to Table III.

relatively low as sheep placenta may not be equally permeable to individual maternal unesterified fatty acids (4). Fetal sheep in fact have very low concentrations of plasma unesterified fatty acids (14). However, within the first few hours after birth this fraction increases considerably (14) and it has been suggested that unesterified fatty acids are being rapidly mobilized from body stores to supply the energy needs of the newborn. Although the cause of this sudden increase is as yet unknown, some workers suggest that it may be associated with a sudden increase in sympathetic nervous activity resulting in subsequent catecholamine release (4,14). Intravenous infusions of either noradrenaline or adrenaline have a marked adipokinetic effect (15,16).

The rapid rise in concentration of plasma lipids during the first two days after birth in the lambs receiving the natural diet resulted from the high intake of fat during this period provided by the ewe colostrum and milk. The colostrum of the ewe has been shown to contain about 18% fat (8) but the fat content of the milk declines rapidly to 10% by the fourth day after parturition. The decrease that occured in lipid concentration in the plasma of the naturally-fed lambs during the later part of the experiment is presumably a reflection of the decreased fat content in the ewes' milk.

It has already been shown by Leat (17) that the fatty acid composition of the newborn ruminant plasma differs markedly from that of the adult. The plasma of the newborn ruminant contains negligible concentrations of the C_{18} polyunsaturated fatty acids whereas the plasma of the adult ruminant contains relatively high concentrations of the C_{18}, C_{20} and C_{22} polyunsaturated fatty acids. These polyunsaturated fatty acids in the plasma of the adult are mainly confined to the cholesteryl ester and phospholipid fractions, which together account for some 75-80% of the plasma lipids of sheep (12). As the only possible source of these acids in the newborn animal is from the unesterified fatty acids of the mother's circulation, the absence of these acids from the unesterified fatty acids fraction of the adult ruminant plasma accounts for the very low concentration of the higher polyunsaturated fatty acids in the blood of the newborn ruminant.

The present experiment shows that in plasma phospholipids of the newborn lamb there are substantial amounts of C_{20} and C_{22} polyunsaturated fatty acids but virtually no linoleic or linolenic acids. The work of Scott et al. (18) on the phospholipid composition of ovine fetal lipids showed that, although there were very low concentrations of linoleic acid and

TABLE VI

Fatty Acid Composition of the Plasma Phospholipids From the Lambs Receiving the Natural Diet and the Artificial Low Fat Diet[a]

Days after birth	16:0			16:1			18:0			18:1			18:2		
	Natural	LS	Low fat	Natural	LS	Low fat	Natural	LS	Low fat	Natural	LS	Low fat	Natural	LS	Low fat
0	30.0		27.1	5.95^{c}		5.42	13.6		14.6	25.8		24.9	1.1		1.5
1	21.4^{c}	$P<0.05$	25.2	1.58^{c}	$P<0.01$	3.83^{e}	15.7		18.2^{e}	33.6^{c}		32.7^{c}	10.1^{c}	$P<0.01$	2.3
2	22.7^{c}		23.8^{e}	1.08^{c}	$P<0.001$	4.35	19.8^{c}		16.1	33.4^{c}		28.0	12.0^{c}	$P<0.01$	2.5
3	20.4^{c}		23.1^{e}	0.90^{c}	$P<0.001$	4.43	18.9^{c}		18.7^{d}	31.3^{d}		32.7^{c}	15.1^{c}	$P<0.001$	2.7
4	19.8^{c}		22.2^{d}	1.08^{c}	$P<0.001$	3.55^{e}	19.3^{c}		16.8	27.0	$P<0.05$	36.0^{c}	17.6^{c}	$P<0.001$	2.4
6	20.4^{c}		19.8^{c}	0.93^{c}	$P<0.001$	3.95	20.8^{c}		16.8	26.0	$P<0.05$	34.4^{c}	16.2^{c}	$P<0.001$	2.8
8	20.5^{c}		22.9^{d}	1.28^{c}	$P<0.001$	4.03	20.5^{c}		16.5	24.9	$P<0.05$	32.8^{c}	15.0^{c}	$P<0.001$	2.1
SE within treatment between days		±1.58			±0.688			±1.40			±1.92			±1.25	
SE between treatment within days		±1.58			±0.688			±1.69			±2.88			±1.47	
	18:3			20:3$\Delta^{5,8,11}$			20:4$\Delta^{5,8,11,14}$			22:5			22:6		
0	Trace		Trace	5.15		4.30	5.35		5.42	2.93		2.83	5.20		5.60
1	1.30^{d}		0.18	3.93^{c}		3.30^{c}	3.70^{c}	$P<0.05$	4.50^{d}	0.95^{c}	$P<0.01$	2.28^{e}	3.20^{c}		3.50^{c}
2	2.12^{c}	$P<0.05$	Trace	0.25^{c}	$P<0.001$	3.93^{e}	3.58^{c}	$P<0.001$	4.98	0.53^{c}	$P<0.001$	2.53	1.05^{c}	$P<0.001$	6.58^{c}
3	2.83^{c}	$P<0.01$	Trace	0.63^{c}	$P<0.001$	4.73	4.08^{c}	$P<0.01$	3.15^{c}	0.10^{c}	$P<0.001$	2.83	1.30^{c}	$P<0.001$	5.05^{e}
4	2.28^{c}	$P<0.01$	0.15	0.53^{c}	$P<0.001$	4.50	4.73^{e}	$P<0.001$	2.55^{c}	1.95^{c}	$P<0.001$	4.13^{c}	2.03^{c}	$P<0.05$	2.85^{c}
6	3.15^{c}	$P<0.01$	0.20	0.35^{c}	$P<0.001$	6.65^{c}	4.95	$P<0.001$	2.90^{c}	0.10^{c}	$P<0.001$	3.20	1.85^{c}	$P<0.001$	6.68^{c}
8	2.50^{c}	$P<0.01$	Trace	0.50^{c}	$P<0.001$	8.95^{c}	6.75^{c}	$P<0.001$	3.13^{c}	0.98^{c}	$P<0.001$	2.80	2.80^{c}		3.38^{c}
SE within treatment between days		±0.44			±0.286			±0.343			±0.164			±0.271	
SE between treatment within days		±0.55			±0.358			±0.343			±0.211			±0.304	

[a]Major components, weight percentage of total. For statistical treatment see footnote to Table III.

TABLE VII

Fatty Acid Composition of the Plasma Cholesteryl Esters From the Lambs Receiving the Natural Diet and the Artificial Low Fat Diet[a]

	16:0			16:1			18:0			18:1		
Days after birth	Natural	LS	Low fat	Natural	LS	Low fat	Natural	LS	Low fat	Natural	LS	Low fat
0	35.1		32.6	12.88		10.60	6.00		6.45	39.7		39.3
1	26.7[d]	$P<0.05$	31.9	5.58[c]		8.95	8.23		6.80	48.3[e]		45.9
2	23.5[c]	$P<0.05$	28.4	4.80[c]	$P<0.05$	8.73	4.03	$P<0.05$	7.65	49.5[e]		43.4
3	19.9[c]	$P<0.01$	28.4[e]	3.63[c]	$P<0.05$	9.10	3.35		5.78	47.1		49.5[e]
4	20.1[c]	$P<0.01$	26.8[d]	3.28[c]	$P<0.01$	9.95	4.08		6.25	45.1		48.7[e]
6	17.9[c]	$P<0.01$	25.3[c]	2.33[c]	$P<0.01$	9.13	4.38		6.48	46.5		52.3[c]
8	19.3[c]	$P<0.05$	25.1	2.55[c]	$P<0.01$	8.98	3.98	$P<0.05$	7.65	42.0		48.2[e]
SE within treatment between days		±2.56			±1.298			±1.449			±4.36	
SE between treatment within days		±2.56			±1.601			±1.463			±4.79	
	18:2			18:3			20:3$\Delta^{5,8,11}$			20:4$\Delta^{5,8,11,14}$		
0	2.60		3.00	0.55		0.51	1.42		1.57	1.22		1.15
1	6.65[e]		2.50	0;20		Trace	Trace[c]	$P<0.001$	1.18	0.60[c]	$P<0.001$	Trace[c]
2	14.58[c]	$P<0.01$	3.88	2.47[c]	$P<0.05$	Trace	Trace[c]	$P<0.001$	1.49	0.58[c]	$P<0.001$	Trace[c]
3	18.50[c]	$P<0.01$	2.53	3.80[c]	$P<0.01$	Trace	Trace[c]	$P<0.001$	1.62	0.31[c]	$P<0.001$	Trace[c]
4	22.22[c]	$P<0.01$	4.30	4.16[c]	$P<0.01$	Trace	Trace[c]	$P<0.001$	1.32[d]	0.57[c]	$P<0.001$	Trace[c]
6	23.40[c]	$P<0.001$	3.55	5.05[c]	$P<0.01$	Trace	Trace[c]	$P<0.001$	1.09[c]	0.42[c]	$P<0.001$	Trace[c]
8	26.20[c]	$P<0.001$	6.28	4.77[c]	$P<0.01$	Trace	Trace[c]	$P<0.001$	1.04[c]	0.37[c]	$P<0.001$	Trace[c]
SE within treatment between days		±2.130			±0.456			±0.079			±0.040	
SE between treatment within days		±2.554			±0.611			±0.101			±0.047	

[a]Major components, weight percentages of total. For statistical treatment see footnote to Table III.

linolenic acid in the fetal liver, the proportion of arachidonic acid accounted for about 11% of the total fatty acids present; this suggests that there may be a rapid conversion of the C_{18} polyunsaturated fatty acids into C_{20} or higher polyunsaturated fatty acids by the fetal tissues. The presence of $\Delta^{5,8,11}$ eicosatrienoic acid in the plasma of newborn lambs has also been noted by Leat (17); it would appear that the fetal ruminant receives insufficient amounts of linoleic acid during development to prevent the accumulation of this metabolite which is normally accepted as being a characteristic of essential fatty acid deficiency (19,20). Investigations with monogastric animals under varying degrees of essential fatty acid deficiency suggest that a triene-tetraene ratio greater than 0.4 in various tissues, including blood plasma, is indicative of a dietary deficiency of linoleic acid (21-23).

In the present experiment there were large increases in concentrations of linoleic acid in plasma phospholipids and cholesteryl esters of naturally-fed lambs after birth. In the phospholipid fraction this was accompanied by a decrease in concentration of $\Delta^{5,8,11}$ eicosatrienoic acid and a resultant decrease in the triene-tetraene ratio. Work on various non-ruminants (21,24-26) has indicated that the minimum dietary requirement of linoleic acid to prevent symptoms of essential fatty acid deficiency is 1% of the total caloric intake. Using results obtained by Barnicoat et al. (27) for the composition of ewes' milk during the first week of lactation it may be calculated that linoleic acid should comprise at least 1.5-1.7% of the total milk fatty acids to satisfy this minimum essential fatty acid requirement. In spite of the large increase observed in the concentration of linoleic acid in the plasma of naturally-fed lambs, analysis of the ewes' colostrum and milk immediately after parturition (8) showed that essential fatty acid concentration in the milk fat was always less than 1% thus the requirements suggested for monogastric animals were certainly not satisfied. From our results these values for essential fatty acid requirements do not appear to be applicable to young ruminants.

In spite of the small concentration of 18:2 in ewes' milk (8) the intake of 18:2 by the suckling lamb is apparently more than sufficient to account for the pronounced increase in plasma lipid fractions that occurs during the first three days after birth. From the results of Thomson and Thomson (28) the total intake of milk by the suckling lamb during the first three days of life is about 1800 g. This corresponds to an intake of 1.56 g 18:2 over the first three days. From estimates of plasma volume and plasma lipid composition, the total amount of 18:2 circulating in the plasma increased by about 70 mg during the first three days. However, changes in the 18:2 content of other tissues during this period must also be taken into account before the balance of 18:2 in the young lamb is fully understood.

Although it cannot be claimed that lambs maintained on the artificial diet in the present trial were exhibiting the external physical abnormalities seen in essential fatty acid deficiency, the progressive increase in the triene-tetraene ratio that occurred in the plasma lipids of these animals, together with the general lack of well-being and poor physical condition that developed over the eight days of the experiment (Table II), does correlate with this condition.

The concentration of linoleic acid in plasma phospholipids of naturally-fed lambs increased nearly tenfold within the first 24 hr after birth, whereas in the cholesteryl esters it only doubled. However, by the third day, the linoleic acid in the cholesteryl ester fraction was greater than that in phospholipids, and by the end of the eight day it accounted for some 26% of the total fatty acids present in cholesteryl esters as compared with 15% in phospholipids. Although the greatest increases in linoleic acid occurred in the cholesteryl ester and phospholipid fractions, by the end of the experiment concentrations of linoleic acid in the triglyceride and unesterified fatty acid fractions had also undergone significant increases. Incorporation of linoleic acid into various plasma lipid fractions of the lamb appears to be similar to that in adult sheep (5). In the adult it was suggested that hydrolysis of chylomicron triglycerides in the liver is followed by an initial and preferential utilization of linoleic acid in the synthesis of plasma phospholipids; this is then followed by incorporation of linoleic acid into plasma cholesteryl esters. Specificity of the enzyme system in the liver is such that little of the liberated linoleic acid is utilized for resynthesis of triglycerides. Although the origin of linoleic acid utilized for cholesteryl ester synthesis is obscure, some enzymic transfer of linoleic acid from the β-position of plasma phosphatidyl choline to cholesterol may occur.

This process has been demonstrated to occur in human plasma (29). It has already been shown by Noble et al. (30) that phosphatidyl choline comprises about 80% of circulatory phospholipids of the cow and contains high concentrations of linoleic acid.

At birth all the plasma lipid fractions contained high levels of palmitoleic acid, in

particular, cholesteryl esters in which palmitoleic acid accounted for some 12% of the total fatty acids present. These levels were maintained throughout the whole experimental period in artificially-fed lambs, whereas in lambs receiving the natural diet, the concentration of palmitoleic acid was markedly reduced within 24 hr of birth. High concentration of palmitoleic acid in plasma lipids at birth presumably reflects dependence of the fetus during development upon de novo synthesis of fatty acids by the soluble enzyme system and desaturation of palmitic acid thus produced (31). With the intake of large concentrations of exogenous lipid from the diet it would be expected that de novo synthesis would be severely curtailed and the formation of palmitoleic acid reduced accordingly (31). A decrease in concentration of palmitoleic acid in tissues of rats after birth has been noted by Sand et al. (32), whereas rats maintained on an essential fatty acid deficient diet increased their content of palmitoleic acid during the development of deficiency. Mead (33) has also demonstrated a high concentration of palmitoleic acid in the adipose tissues of rats given a fat deficient diet and suggested that synthesis of large amounts of palmitoleic acid is an attempt by the animal to maintain desirable properties of body lipids ordinarily achieved by dietary unsaturated acids such as linoleic acid. It is also interesting to note that in the work of Moore et al. (34) in which cows were fed a diet containing 10% palmitic acid the plasma showed increased concentrations of palmitoleic acid which appeared to be preferentially incorporated into the cholesteryl ester fraction.

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Determination of Plasma Tocopherols by Gas Liquid Chromatography

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ABSTRACT

A method is described for the separation and determination of plasma tocopherols by gas liquid chromatography (GLC). Proteins in 0.1 g samples of plasma were precipitated with ethanol containing a known amount of 5,7-dimethyltocol which served as an internal standard. Tocopherols were extracted into petroleum ether, purified by thin layer chromatography and analyzed as trimethylsilyl ethers by gas liquid chromatography (GLC) on 0.5% Apiezon L. Recoveries of α- and γ-tocopherols averaged 100% and 93%, respectively. The mean total tocopherol content of eight human plasma samples was 8.5 μg/g by GLC and 9.9 μg/g by a ferric chloride-α,α'-dipyridyl method. The α- and γ-tocopherol contents of 16 human plasma samples ranged from 4.0 to 12.3 μg/g and 0.6 to 2.1 μg/g, respectively.

INTRODUCTION

An evaluation of the vitamin E potency of natural products is complicated by the differences in biological activity of the various tocopherols (1). Although α-tocopherol, the most biologically active member of the series, is of greatest interest, it is usually found in mixtures with other tocopherols. In blood plasma, γ-tocopherol is usually present and can be a significant part of the total tocopherol (2). Since forms other than α-tocopherol are present, a method is needed for measuring the vitamin E content of plasma that is specific for individual tocopherols. For routine analysis, this method should be relatively simple and able to be used with small samples.

Present methods for plasma vitamin E generally require extraction of tocopherols into a hydrocarbon solvent followed by spectrophotometric analysis of the extract with a correction for carotenoids (3-5). These methods are adequate for many purposes but do not differentiate among tocopherols and are subject to interferences from other reducing substances. Many investigators have removed interfering substances and separated the various tocopherols by paper (6,7), column (6), or thin layer chromatography (TLC) (8) before spectrophotometric analysis. No reference to methods that apply these chromatographic techniques to plasma sample sizes of less than 1 ml has been found.

A previous publication from this laboratory reported a gas chromatographic method for the separation and quantitation of tocopherols in foods (9). The present paper describes the modification and adaptation of that method to the analysis of individual tocopherols in 0.1 g samples of plasma or serum.

EXPERIMENTAL PROCEDURES

Operating conditions and materials were essentially the same as previously described (9).

Apparatus

The gas chromatograph was an F&M Model 810 modified to reduce dead volume and to provide for on-column injection. The coiled

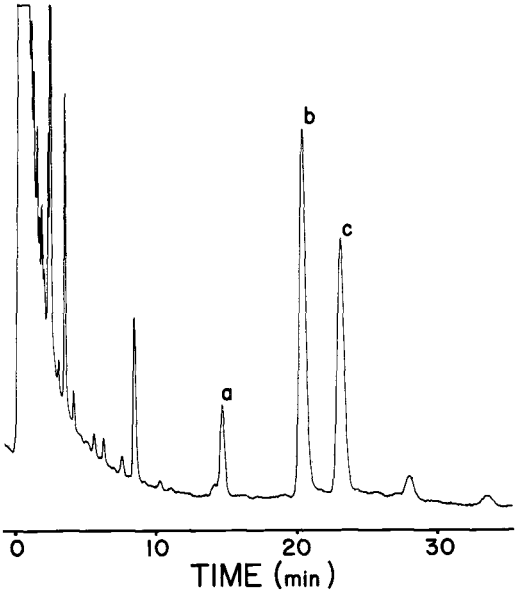

FIG. 1. Gas chromatographic separation of human plasma tocopherol TMS ethers on 0.5% Apiezon L: a, γ-tocopherol; b, 5,7-dimethyltocol (internal standard); c, α-tocopherol. Chromatographic conditions are described in the text.

TABLE I

Recoveries of Total Tocopherol[a] From 0.1 g Plasma

Data	Sample 1		Sample 2	
	Alpha	Gamma	Alpha	Gamma
μg tocopherol/0.1 g plasma	1.04	0.19	0.40	0.10
μg tocopherol added	1.06	0.80	1.06	0.80
Range rec., %	97-106	83-95	98-103	100-102
av. rec., %	100	90	100	101
No. detn.	9	9	3	3

[a]Total tocopherol equals sample tocopherol plus added α- and γ-tocopherol.

glass columns were 0.125 in. OD x 15 ft long, silanized and packed with 0.5% Apiezon L on 100/120 mesh Gas Chrom Q. All separations were isothermal at 250 C. The injection port was also maintained at 250 C and the flame ionization detector at 280 C. The carrier gas was helium at a flow rate of approximately 25 ml/min, preheated to 250 C. The electrometer was normally operated at 1.6×10^{-11} amp full scale (range 1, attenuation 4 on the Model 810).

Special tubes for handling 1 ml and 10 μl volumes were made from disposable Pasteur pipets (Fisher Scientific Co.). New pipets were soaked in cleaning solution, rinsed thoroughly with distilled water and oven dried at 110 C. The clean tubes were sealed at the point where the capillary was drawn from the body of the pipet to produce a tube approximately 4.5 in. long with a narrow tip. Small volumes in the tip could be reached with a syringe needle by breaking off the top of the tube.

TABLE II

Tocopherol Content[a] of Human Blood Plasma

Subject No.	Alpha, μg/g	Gamma, μg/g
1	12.3 ± 0.87	1.5 ± 0.64
2	11.0 ± 0.55	1.2 ± 0.25
3	10.7 ± 0.40	2.1 ± 0.36
4	8.9 ± 0.49	0.7 ± 0.06
5	8.5 ± 0.29	1.5 ± 0.21
6	8.0 ± 1.72	1.0 ± 0.30
7	7.9 ± 1.54	0.8 ± 0.15
8	7.1 ± 0.66	1.2 ± 0.40
9	6.5 ± 0.44	0.6 ± 0.10
10	6.7 ± 0.58	1.7 ± 0.17
11	6.4 ± 0.93	1.6 ± 0.23
12	6.1 ± 0.68	1.3 ± 0.31
13	6.1 ± 0.52	0.7 ± 0.15
14	5.8 ± 0.80	1.1 ± 0.12
15	4.9 ± 0.74	0.7 ± 0.15
16	4.0 ± 0.72	1.1 ± 0.10

[a]Average and standard deviation for samples drawn on three consecutive days.

Internal Standard

5,7-Dimethyltocol (Pierce Chemical Co.) in ethanol (10 μg/ml) was used as internal standard at a concentration approximating levels of α-tocopherol expected in plasma. Pyrogallol (2 mg/ml) was added as an antioxidant.

Thin Layer Chromatography

Silica gel (Silica Gel HR extra pure acc. to Stahl, Brinkmann Instruments, Inc.) plates 250 μ thick were air dried for 1 hr, heated at 110 C for 1 hr, and stored until needed in a desiccator. Just before use they were predeveloped with methanol, marked off into 2 cm strips and activated at 110 C for 15 min. Developing chambers lined with filter paper were prepared with a solvent system of 0.75% methanol in benzene and allowed to equilibrate for 1 hr.

Method

Plasma or serum (90-120 μl) measured roughly with a disposable bore pipet (Microtrol, Drummond Scientific Co.) was transferred to a 2 ml conical centrifuge tube and weighed. Exactly 100 μl of 5,7-dimethyltocol internal standard in ethanol was added and the sample mixed thoroughly. Petroleum ether (200 μl; Mallinckrodt Chemical Works, nanograde) was added, the tube stoppered with a polyethylene stopper and then mixed vigorously on a vibrator for 30 sec. After centrifugation, the petroleum ether was transferred to a 2 or 3 ml centrifuge tube and evaporated to dryness with a gentle stream of nitrogen. The residue was dissolved in 20 μl of petroleum ether and applied to a 2 cm strip on a thin layer plate. A 10 μl petroleum ether rinse of the tube was added to the same spot. A standard reference mixture containing approximately 10 μg each of α- and δ-tocopherol was spotted on the two outside strips and the plate was developed for 10 cm. The side strips bearing the reference standard were sprayed with phosphomolybdic acid to determine the limits of tocopherol travel. The corresponding tocopherol area

TABLE III

Comparison of Methods

	GLC				Chemical[a]	
Subject No.	Alpha, μg/g	Gamma, μg/g	Total, μg/g	No. detn.	Total, μg/g	No. detn.
1	12.9	1.2	14.1	2	9.5	2
2	4.6	1.2	5.8	2	7.7	2
3	4.1	0.9	5.0	2	8.2	2
4	5.8	0.9	6.7	2	6.7	2
5	7.0	1.8	8.8	2	12.9	2
6	8.6	0.8	9.4	2	11.4	2
7	6.1	0.6	6.7	2	8.5	2
8	10.4	1.9	12.3	9	13.9	6

[a]Bieri et al. (5).

bearing the sample was scraped from the plate and transferred to a 3 ml centrifuge tube containing 1 ml of methanol (99.9 Mol % pure, Fisher Scientific Co.), mixed well, and centrifuged. The supernatant solution was decanted into a sealed Pasteur pipet and solvent was evaporated with a stream of nitrogen. The pipet was rinsed down with a small amount of methanol, the top was broken off to leave a sealed bottom section less than 2 in. long, and the solvent was evaporated completely. The thoroughly dried sample residue was dissolved in 10 μl of a mixture of hexamethyldisilazane, trimethylchlorosilane, and dry pyridine (9:6:10), re-evaporated to dryness and dissolved in 10 μl of carbon disulfide. A 2 μl aliquot sandwiched in the syringe between 1 μl plugs of carbon disulfide was injected on the gas chromatograph. Peak areas were measured by triangulation.

The amount of tocopherol in micrograms per gram was equal to $(W_i/A_i) \times (A_t/W_s)$, where W_i is the weight of added internal standard in micrograms, A_i the area of the internal standard peak, A_t the area of the tocopherol peak, and W_s the weight of the sample in grams.

RESULTS AND DISCUSSION

Precision

A series of nine analyses of the same citrated plasma sample, made in sets of three on three different days, gave a coefficient of variation of 4.5% for α-tocopherol and 5.8% for γ-tocopherol. The average α- and γ-tocopherol values for this sample were 10.4 and 1.9 μg/g, respectively. This variation of the method was compared with daily variation of the gas chromatograph. Aliquots (100 μl) of a standard mixture of 10.6 μg/ml α-tocopherol and 8.1 μg/ml γ-tocopherol and 5,7-dimethyltocol in ethanol were evaporated, derivatized and injected on the gas chromatograph on 10 different days. Under these conditions, coefficients of variation were 4.2% for α-tocopherol and 5.6% for γ-tocopherol.

Recoveries

An ethanolic solution containing 1.06 μg α-tocopherol, 0.80 μg γ-tocopherol and 5,7-dimethyltocol internal standard was added to 0.1 g subsamples of two plasmas. Recoveries for the total tocopherol, added plus sample, are

TABLE IV

Stability of Blood Tocopherols During Storage at -40 C

		Plasma					
		EDTA		Oxalate		Serum	
Subject No.	Time, wk	Alpha, μg/g	Gamma, μg/g	Alpha, μg/g	Gamma, μg/g	Alpha, μg/g	Gamma, μg/g
1	0	12.5	3.9	13.4	3.5	13.7	3.9
	4	12.7	4.1	12.6	3.5	15.3	3.7
	8	12.1	3.9	12.1	3.6	12.3	3.9
2	0	3.7	1.1	4.0	1.0	4.6	1.1
	4	3.8	1.0	4.2	0.9	3.8	1.1
	8	4.6	1.2	3.7	1.2	4.1	1.2

shown in Table I. Average recoveries for 12 determinations were 100% for α-tocopherol and 93% for γ-tocopherol. These acceptable recoveries from plasma, relative to 5,7-dimethyltocol, were in contrast to 80-85% recoveries when water was substituted in the method for plasma. A systematic examination of all steps in the procedure, including the effects of extraction and susceptibility to destruction from each solvent, showed these low recoveries were due to losses during TLC. The amounts chromatographed were 1.2 μg of α-tocopherol and 0.85 μg of γ-tocopherol; recoveries of each averaged 83%. These findings suggest that tocopherols from plasma suffer less destruction than the pure tocopherol standards, perhaps because of the presence of other lipid material. If this is true, the use of standard curves or the calculation of recoveries based on TLC of pure standards may be suspect.

Tocopherols in Human Plasma

As a test of the applicability of the method, blood from a series of human donors was analyzed for tocopherols. Samples were drawn from 16 donors, following a 12 hr fast, on each of three consecutive days and the oxalated plasma was stored at -40 C until analyzed. Duplicate analyses were made on each sample and values for three days were averaged (Table II). Only α- and γ-tocopherols were detected with ranges of 4.0-12.3 μg/g and 0.6-2.1 μg/g, respectively. The total tocopherol ranged from 5.1-13.8 μg/g with an average of 8.7 μg/g.

Comparison of Methods

Eight human plasma samples were analyzed by both GLC and a modified Emmerie-Engel colorimetric method (Table III). The method chosen for comparison was that of Bieri et al. (5), with minor volume changes to suit available cell sizes; this method does not require a TLC step. The only sample which gave a lower result by the colorimetric method was slightly lipemic. One sample gave the same results by both methods while the other six samples were all higher by the colorimetric method. The higher values by the colorimetric method are probably due to other reducing substances.

Stability During Storage

To test the stability of blood tocopherols during storage, plasma (EDTA and oxalate) and serum from two different donors were analyzed immediately and then subsamples were analyzed again after four and eight weeks of storage at -40 C. All samples showed no significant change after eight weeks of storage (Table IV).

Impurities

Only solvents of high purity could be used; these were selected to give minimum interference on the gas chromatogram. Commercial products of adequate purity were available for most solvents, others were purified by distillation. Several extraneous GLC peaks derived from silica gel were reduced to trace levels by using Silica Gel HR. Incomplete separation of one of these impurities from γ-tocopherol interfered with the determination of trace amounts of this tocopherol. To minimize this difficulty, TLC plates were predeveloped in methanol for at least 10 cm just prior to use and samples were developed on 2 cm strips. Because of possible contamination from the atmosphere, plates were used within three days of spreading.

Internal Standard

5,7-Dimethyltocol met several criteria which made it a suitable internal standard for the determination of other tocopherols. On TLC with the methanol-benzene system described, its R_f was similar to that of α-tocopherol, and on GLC it emerged just before α-tocopherol with complete separation. There were further advantages in the selection of 5,7-dimethyltocol in that it does not occur in natural products and is available commercially.

Since α-tocopherol was the purest tocopherol available (>99%), the 5,7-dimethyltocol solution was standardized against α-tocopherol by chromatographing mixtures of these two tocopherols. This standardized value for 5,7-dimethyltocol, therefore, included any correction factor necessary for calculating α-tocopherol. Both β- and γ-tocopherol had flame ionization detector responses of 1.0 relative to the standardized 5,7-dimethyltocol and did not require a correction factor in the calculation.

Gas Chromatography

Under the conditions described, retention times were approximately 15 min for γ-tocopherol, 21 min for 5,7-dimethyltocol, and 24 min for α-tocopherol. Columns had efficiencies in excess of 5500 theoretical plates.

The limiting factor determining the number of samples that could be handled in one day was the gas chromatograph. With one analytical column we found eight samples could be handled conveniently with simultaneous quantitation of each tocopherol.

With care in transferring solvents 0.3 μg γ-tocopherol per gram plasma and 0.6 μg α-tocopherol per gram plasma could be measured in 0.1 g samples. This lower limit of sensitivity is due in part to interferences from impurities derived either from solvents or from

silica gel. Elimination of these trace impurities would probably make this method sensitive to lower amounts of tocopherols.

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Mycobacterial Sulfolipids: Spontaneous Desulfation

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ABSTRACT

A glycolipid sulfate elaborated in abundance by surface-grown cultures of *Mycobacterium tuberculosis* has been characterized in earlier studies as a complex 2,3,6,6'-tetraacyl α, α' trehalose 2'-sulfate. The purified ammonium salt undergoes rapid quantitative desulfation on mere dissolution in reagent grade anhydrous ether. The same reaction is observed in sulfolipids recovered from a number of human strains and one bovine strain. Experimental observations on the normal and acid-catalyzed rates of the reaction and on the influence of other solvents and other cationic forms support a proposed mechanism, which is detailed. Structural criteria which may influence the proclivity for spontaneous desulfation are discussed.

INTRODUCTION

Surface cultures of virulent strains of *Mycobacterium tuberculosis* var. *hominis* were found by Middlebrook et al. to elaborate a strongly anionic sulfur-containing lipid which, Middlebrook suggested, might play a prominent role in the biology of tuberculosis (1,2; also unpublished data). My studies, begun in collaboration with Middlebrook, have shown that several families of sulfated lipids are produced by the strain H37Rv. The most abundant member is designated as sulfolipid I (SL-I) and comprises a group of very closely related 2,3,6,6'-tetraacyl trehalose esters in which the carbohydrate bears a single equatorial sulfate half ester in the 2'position, a rarely sulfated position in natural carbohydrate sulfatides. Recovered as the ammonium salt in our purification scheme, NH_4SL-I has a molecular weight of approximately 2400 and an average empirical formula and gross structure as depicted in Figure 1 (3,4).

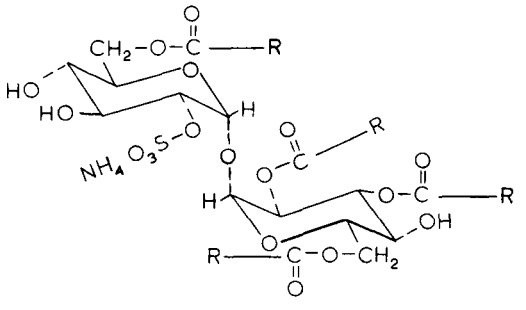

FIG. 1. Ammonium Sulfolipid-I of *M. tuberculosis*; approximate molecular formula $C_{145}H_{283}O_{20}NS$.

Desulfation of NH_4SL-I

NH_4SL-I, and indeed several other of the mycobacterial SL groups, exhibit a remarkable propensity for desulfation under mild, nondegradative conditions. On mere dissolution of small amounts of NH_4SL-I in reagent grade anhydrous ether, complete desulfation occurs in a short time at room temperature. Apparently a spontaneous hydrolytic process, the necessary water for the hydrolysis of a substance of the indicated molecular weight is more than adequately provided for by that already present in the solvent. The desulfation of mycobacterial NH_4SL-I results in the release of a mole of NH_4HSO_4 and restoration of the original hydroxylic function, i.e., with retention of configuration. This was assured in the following specific experiments: the carbohydrate sulfate from gentle alkaline solvolysis of SL-I affords trehalose on hydrolysis in aqueous acid at room temperature; similarly the spontaneously desulfated lipid gives trehalose quantitatively on alkaline solvolysis.

Although alkali- or acid-catalyzed hydrolysis of carbohydrate sulfates has been extensively described in aqueous systems (5), with only occasional exceptions (6-8), the desulfation process of the present is more similar to solvolytic transformations which had been described solely for certain steroid sulfates (9-11) and were only recently extended to simpler alcohols (12). Our accidental rediscovery of this facile reaction in the domain of the mycobacterial sulfolipids yielded an independent mechanistic interpretation which differs in some respects from those previously advanced, as in the review of Haines (13).

Proposed Mechanism

Because the spontaneous desulfation of NH_4SL-I in ether proceeded with retention of configuration and was dramatically catalyzed by traces of mineral acid, we reasoned that except for rate, the solvolytic behavior of

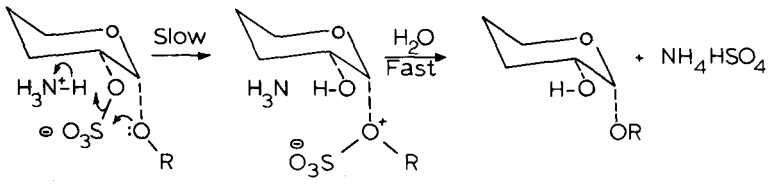

FIG. 2. Proposed mechanism for desulfation of NH_4SL-I. Overlap of electrons from the anomeric oxygen into the sulfur d orbital facilitates attack of the ester oxygen by the proton (or NH_4 ion). The intermediate oxonium salt is then hydrolyzed.

NH_4SL-I resembled the hydrolytic behavior of simpler carbohydrate sulfates (5) or of acid-catalyzed desulfations in methanol (6-8). We postulated that in an aprotic but yet sufficiently basic solvent, e.g., ether, the ammonium ion can yield, or hydrolyzes to yield, a proton to coordinate with the ester oxygen which links the carbohydrate moiety to the sulfur and thus provides the hydrolysis susceptible transition state. The apparent spontaneity and rapidity of the reaction is probably accounted for by the fact that the energy barrier to this attack is lowered, by the proximity of the anomeric oxygen (α-configuration) to the sulfate substituent in the adjacent equatorial position. With the glucose moiety in the very probable C-1 conformation, the anomeric oxygen is separated from the oxygen at the 2 position by about 2.85 A (14). In a Fisher-Hirschfelder-Taylor model construction, the anomeric oxygen and the sulfur are, in fact, in contact. As a first step (Fig. 2) we suggest the overlapping of an electron pair from the oxygen into the *d*-orbitals of the S atom to relieve the polarization on the ester oxygen and to make a pair of electrons more available to attack by the proton, a slow but possibly concerted step. Followed by the necessary intervention of a mole of water, scission occurs to release a mole of NH_4HSO_4 and the desulfated lipid.

A system conforming to this model should have the following characteristics: (a) the reaction rate should be autoaccelerating, since each scission yields an additional proton. As a trivial corollary, desulfation should be catalyzed by mineral acids. (b) Other appropriate solvents should promote desulfation, e.g., acetone. (c) Protogenic, more basic solvents, e.g., water or alcohols, added to the ether should tend to inhibit the reaction. (d) "Onium" nitrogen salts bearing a proton should desulfate spontaneously; however, simple metal salts should not. These requirements follow logically from the thesis which is advanced. They are not entirely in accord with results described with steroid sulfates, e.g., reported desulfations of alkali metal salts (11). However, with the precautions taken in our experiments, all of the results were realized.

METHODS

Early direct qualitative evidence substantiated the fact that complete desulfation of dilute solutions of NH_4SL-I in ether occurs in less than 5 min on the addition of appropriate, small quantities of sulfuric acid. Therefore, to minimize chance contamination by catalytic traces of acid, all glassware employed in desulfation studies was heated in dilute alkali, exhaustively rinsed and oven dried. This precaution does not seem to have been anticipated in earlier studies.

To study the time course of desulfations, radiolabeled (^{35}S) NH_4SL-I of known specific activity was dissolved in appropriate quantities of Baker's reagent grade anhydrous ether (H_2O content .008-.01%) along with any other reagents that were to be tested. The concentration of NH_4SL-I was ordinarily about 0.1-0.3 mmolar at the final dilution. Aliquots were sampled at intervals, the desulfation terminated by quenching the sample with water-saturated ether about 0.5 M in NH_4OH, and hexane soluble radiosulfate recovered (an aqueous Na_2CO_3 extraction is required) and assayed radiometrically. In companion experiments, the quenched samples were examined by thin layer chromatography (TLC) on silica gel (development in $CHCl_3/CH_3OH/H_2O/CH_3COOH$, 95:1:0.3:5).

NH_4SL-I preparations used in the studies were obtained from ammoniacal chromatography effluents and stored in dilute hexane solutions (3). These undergo a very slow spontaneous desulfation; complete desulfation occurs in about two years. Accordingly, some of the experiments were probably conducted with material in which spontaneously generated degradation products were already present in undetectable amounts. As a terminal pretreatment to minimize even these, the hexane solutions of NH_4SL-I were filtered through a

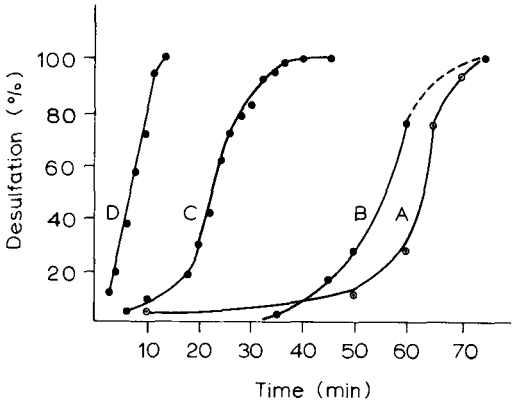

FIG. 3. Time course of NH_4-sulfolipid desulfation in ether. A and B, spontaneous. C and D, catalyzed by .02 and 0.1 equivalent of H_2SO_4, respectively.

column of Na_2CO_3 directly into the appropriate reaction vessels and taken to dryness under nitrogen. One tenth-millimolar solutions of such preparations, in freshly opened reagent grade ether, were left undisturbed and simply tested for complete reaction. Under these conditions, complete desulfation occurred in 6 to 7 hr.

RESULTS

Kinetics

The time courses of desulfation, both spontaneous and under H_2SO_4 catalysis, are depicted in Figure 3. Curves A and B reflect the behavior of two samples of NH_4SL-I which had been stored in hexane, but were not pretreated with Na_2CO_3 as a final step. The lag or induction period before lysis was detectable does not seem to be reproducible and may reflect minute levels of cleavage products already present in the starting material. Once reaction is detectable however, the autoacceleration of desulfation is clear. Figure 4 is a TLC of samples taken at 5 min intervals from a similar desulfation. This vividly shows the increase in amount of desulfated product ($Rf \approx 0.5$) at the expense of SL-I (at the origin). Curves C and D show the behavior of ether solutions of carbonate-filtered NH_4SL-I to which .02 and 0.1 equivalents, respectively, of concentrated H_2SO_4 were added at zero time. Accordingly, the role of protons generated in uncatalyzed desulfations cannot be ignored (see Discussion).

Influence of Solvents

Spontaneous quantitative desulfation occurred in acetone, but required two days rather than a few hours. The change in rate probably reflects the influence of the solvent on the acidity of the solute. Moreover, the reaction-promoting NH_4HSO_4 cleavage product formed in the acetone solution was largely deposited on the walls of the reaction vessel; in ether, it remains colloidally dispersed.

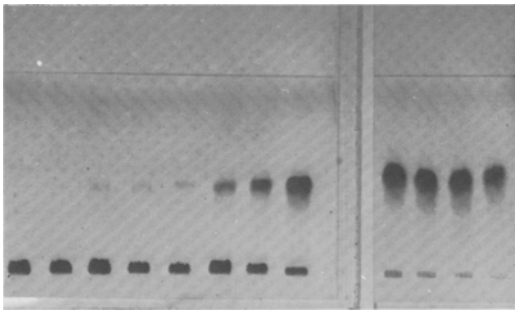

FIG. 4. Silica gel thin layer chromatogram depicting the time course of a normal desulfation of NH_4SL. Sulfolipid remains at origin; desulfated product migrates.

At room temperature, NH_4SL-I proved completely stable in pyridine. Elevated temperatures were not examined. More significantly, 0.5% pyridine prevented the spontaneous desulfation in ether. When moist hexane solutions of NH_4SL-I are heated at reflux, desulfation (and concomitant hydrolysis of acyl functions) can be recognized in a few hours. The desulfation is entirely suppressed by a little pyridine. The oily NH_4SL-I obtained from evaporation of SL-I solutions desulfates on keeping for a few weeks, but this too is prevented by a trace of pyridine.

The influence of hydroxylic solvents was as expected and accords with similar observations of steroid sulfates (9,10), i.e., in 50:50 ethanol-ether, essentially no desulfation was detected in a month. The addition of two equivalents of water (based on NH_4SL-I) appeared to promote the desulfation in reagent ether, but it was almost completely inhibited in ether containing 0.5% H_2O.

Other Salts

Pyridonium sulfolipid was completely desulfated after 70 min in ether solution; but NaSL-I was stable; a product judged to contain a minute amount of residual NH_4SL seemed to be slightly decomposed in ether during 7 hr and then stabilized without further degradation in a week. The apparent slight decomposition may be an artifact. As expected, NaSL-I is desulfated in about 30 min in ether containing appropriate small quantities of concentrated H_2SO_4 (10 mole% based on SL). In an NaSL

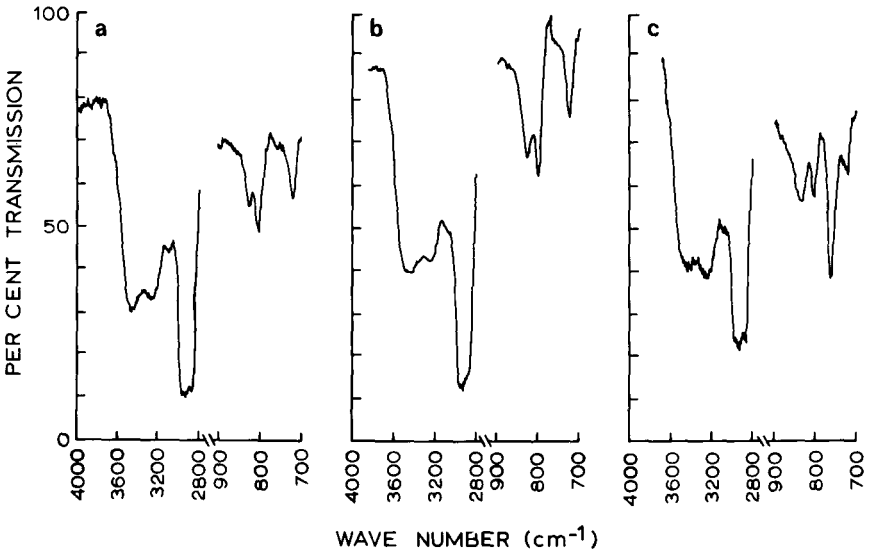

FIG. 5. Partial of IR spectograms, NH_4SL-I, NH_4SL-II, NH_4SL-III. Absorption bands at 3220 and 1400-1450 cm^{-1} confirm that these are ammonium salts. Bands at 808 and 828 (833) cm^{-1} are associated with trehalose and sulfate, respectively.

preparation containing 20% NH_4SL, complete desulfation occurred during 40 hr.

Other Sulfated Lipids, Structural Influences

From some ten Indian and British strains of *M. tuberculosis* and from the bovine strain Ravenel (Goren and Brokl, unpublished data) we have recovered small amounts of purified (radiolabeled) sulfolipids by the chromatographic procedures described earlier. Individual lipids from all of these strains appear to be at least very similar to the principal SL-I of strain H37Rv. All of these SL-I-like substances which have been tested, including the bovine Ravenel sulfolipid were found to desulfate spontaneously in ether.

Minor Sulfolipids of H37Rv

Among the various sulfated glycolipids elaborated by the strain H37Rv and separated in our DEAE chromatographic procedures are minor sulfolipids that have previously been only briefly described (3,4). Table I summarizes certain physical and structural characteristics of these substances, in comparison with SL-I, which have been discerned in studies with the small amounts that have occasionally been available to us.

SL-II and I' desulfated rapidly, but SL-III was entirely stable in ether solution. After 48 hr without detectable change, it was desulfated in a few minutes after the surface of the ether solution was momentarily exposed to a gentle stream of anhydrous HCl gas and the flask restoppered. This is a most gentle catalyzed solvolysis.

Figure 5 (a,b,c) compares the pertinent features of the IR spectra of SL-I,II and III, respectively (the spectrum of SL-I' is essentially the same as that of SL-I). It is clear that all three samples are ammonium salts (NH absorption at 3220 and 1400-1450 cm^{-1}). The absorption at 800-807 is specifically correlated with the trehalose core (4) and is substantially identical in the three spectra. However, for SL-III the sulfate absorption band at 833 cm^{-1} is displaced about 5 to 6 wave numbers from that seen in NH_4SL-I, I' and II (827-28 cm^{-1}), and it is a considerably stronger band in SL-III. This aberration may raise some doubt about the actual position of the sulfate; however, other observations suggest that it is located normally, i.e., at the 2' position. Structural studies indicate that in the other sulfolipids (I, I', II), the sulfate-containing ring is also substituted with a very large acyl function (molecular weight about 550) at the 6' position; in SL-III, this glucose moiety has only the sulfate. The lack of the large acyl function at 6' may account for the displacement in the position and strength of the sulfate absorption band to which we referred; and these consequences may both relate to some conformational change attendant upon the loss of the major acyl substituent. The distinction between NH_4SL-III and the other ammonium sulfolipids, whatever it might be, may in fact be associated with the resistance of the substance to spontaneous desulfation.

TABLE I

Characteristics of Sulfolipids From H37Rv

Sulfolipid	TLC Mobility (relative to SL-I)	Acyl substituents[a]	Position of sulfate	6′ Position
II	1.1	B, 2C, ?	2′	Acylated
I	1.0	A, B, 2C	2′	Acylated
I′	0.9	2A, B, C	2′	Acylated
III	0.33	B, C (?)	2′ (probable)	Vacant

[a]From studies recently completed (4), the acyl substituents identified in these sulfolipids are the following: B, palmitic acid; A, a multi-methyl-branched acid of mean molecular weight 550; C, a hydroxylated derivative of A. The glucose moiety bearing the sulfate is ordinarily referred to as the prime ring; when this carries an acyl function as well, it is apparently only in the 6′ position. These conclusions are based on permethylation studies such as are described by Goren (3).

Brain Sulfatides

Bovine brain sulfatides are cerebrosides sulfated at the 3 position of a galactose moiety (15). Samples from Supelco, examined as received, were resistant to desulfation for months in ether suspension. Other samples were equilibrated with large excesses of ammonium acetate and recovered or were twice adsorbed on DEAE cellulose and recovered by elution with ammoniacal elutrients according to the method of Rouser (16). Products obtained in this fashion were tested in acetone or ether and found to be completely stable. Figure 6 is a silica gel thin layer chromatogram (developed in $CHCl_3/CH_3OH/H_2O$, 65:35:5) in which cerebrosides (sample on left) are compared with material recovered after suspension-dissolution in ether for a week. It is clear that the sulfatides did not degrade.

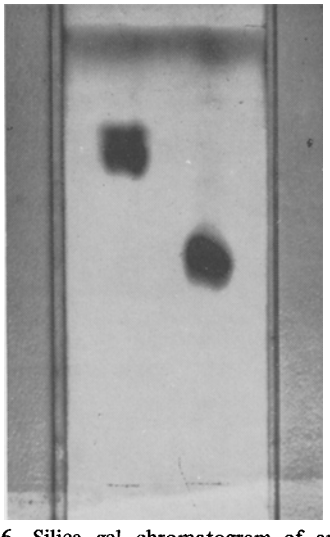

FIG. 6. Silica gel chromatogram of ammonium brain sulfatides after a week in ether (right) as compared with cerebrosides (left).

DISCUSSION

Our results confirm the thesis that glycolipid sulfates are sensitive to hydrolysis in nucleophilic aprotic solvents, particularly in the presence of acid catalysts; moreover, they expand the concept that such catalyzed hydrolysis can occur very rapidly yet gently in essentially anhydrous media. With lipids that are soluble in ether, rapid desulfation may occur spontaneously with appropriately constituted ammonium (or onium) glycolipid sulfates; or the reaction may be promoted through the agency of even traces of mineral acids.

The existence of species of ammonium sulfolipids which do not hydrolyze spontaneously in ether (such as the mycobacterial NH_4SL-III) leads to the important inference that the criterion of ammonium glycolipid sulfate structure is not in itself sufficient to confer proclivity for spontaneous desulfation. Other factors appear to be involved also.

The stability of the brain sulfatides, of NaSL-I (and its destabilization by NH_4SL-I or traces of acid), as well as the stability of NH_4SL-III, support the conviction that the desulfation is a spontaneous one and is not simply the consequence of a chance encounter with a wandering proton. We nevertheless submit that protons probably must be generated to initiate what becomes an irreversible process, the complete desulfation of the sulfatide. Moreover, contamination of a sulfatide salt by a mere trace of the free acid form will probably lead to complete degradation in an appropriate solvent.

The reported ambient temperature desulfation in dioxan of alkali metal salts of steroid sulfates (9,11) does not accord with our findings and conclusions. At present these cannot be reconciled. We are, however, investigating some of these steroid sulfates.

Mayers, et al. (12) and Haines (13) have recently reviewed the solvolysis of steroid sulfates in aprotic nucleophilic solvents and proposed a mechanism for the general reaction which invokes a more definitive role for the small amount of water which participates in the solvolysis. A hydrated state of the sulfatide is postulated, which is attacked and cleaved by the nucleophilic solvent. No role is considered for an electrophile (proton or the like). The well-documented inhibitory effect of larger amounts of water (or alcohol) is explained as a complete hydration (solvation) of the sulfate core so that penetration by the nucleophilic solvent is prevented. This explanation for the curious inhibition of a hydrolysis by excess water is persuasive.

However, the role of protons in the solvolytic process cannot be ignored: note the instability of the free organic hydrogen sulfates (10), the autoacceleration of the spontaneous solvolysis as protons are generated, the solvolysis of stable sulfatides under the influence of minute amounts of acid (6-8), and the total inhibition of the reaction by minute amounts of avid proton binders, e.g., pyridine. It is unlikely that all of these documented multidirectional changes in rate can be attributed solely to favorable or unfavorable alterations in the degree of hydration at the sulfate core.

We hope in a supplemental note to expand and implement this supporting evidence and, accordingly, to develop the thesis that availability of both a quintessential electrophile and nucleophile are necessary for facile solvolysis of the sulfatides.

Studies with the mycobacterial sulfolipids of the present instance have afforded clues suggesting that subtle structural features within the sulfatide (e.g., in SL-III) probably also influence the characteristics of the reaction. From our mechanistic interpretation, in which a probable role is postulated for the α anomeric oxygen, we would expect that corresponding β anomers might show significant differences in their solvolytic behavior. Accordingly, studies of simple analogs may be informative.

Such structural influences might be sought additionally in a careful examination of other appropriate sulfated glycolipids having significantly different structures from those described. The intriguing lipid from *H. cutirubrum* of Kates et al. (8) would be interesting for comparison. Professor Kates informs me that his lipid exhibits marked sensitivity to mineral acids, and indeed desulfation of the lipid for a portion of the structural study (8) was carried out in chloroform, 0.1 M methanolic HCl. However, he has not examined the ammonium salt alone under such conditions as we have detailed. Acid-catalyzed desulfation of the lipids described by the Haines group (12) would also be most informative.

It is well documented that avirulent and attenuated strains of *M. tuberculosis* var. *hominis* produce little or none of the sulfolipids seen in virulent strains (1). Middlebrook's suggestion that this material might have a role in the biology of tuberculosis is partially supported by a subsequent correlation between infectivity and sulfolipid elaboration among a dozen strains which were examined (17). If a teleonomic advantage accrues to the pathogen from its ability to synthesize the sulfolipid, it might well reside in the most unusual propensity for spontaneous desulfation which I have described. Occurring intracellularly in a host, the process would have significant physiological import; a strong mineral acid is released at the site, with the attendant traumatic consequences which this should initiate. A desulfation event taking place in a lipid environment, e.g., a membrane, in which a balanced polar-nonpolar substance is suddenly converted into two fragments of widely differing polarity could severly and adversely affect the structural integrity of that membrane. On the basis of the almost unique suicidal property of onium sulfolipid, the substance is unquestionably endowed with the potential for tissue destruction. This potential manifests itself in demonstrable local toxicity when NH_4SL, but not NaSL, is injected intracutaneously in guinea pigs, an observation which was made long before the spontaneous desulfation was discovered (1). The mysterious difference in toxicity between NaSL and NH_4SL now seems to be entirely explained.

ACKNOWLEDGMENTS

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The Influence of Exogenous Cholesterol on the Fatty Acid Composition of Liver Lipids in the Rats Given Linoleate and γ-Linolenate

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ABSTRACT

Three groups of rats were given cholesterol and cholic acid for four weeks, and then fed a diet with 20% lard (group 1), a diet with 4% of the lard substituted by methyl linoleate (group 2), and a diet with 2% substituted by methyl linoleate and another 2% by methyl γ-linolenate (group 3) during the same period. Two control groups (4 and 5) received the same diets as did groups 2 and 3, respectively, but without cholesterol and cholic acid. The hepatic cholesterol accumulation was significantly less in group 3 than in groups 1 and 2, and distinct differences in the degree of plasma cholesterol elevation were found among the cholesterol fed groups (1>2>3). Supplement of γ-linolenate resulted in a reduction of the ratio of arachidonate to homo-γ-linolenate in both the cholesterol-fed group and the control group, but the ratio was much lower in the former. Incorporation of 1-^{14}C-linoleate and 1-^{14}C-γ-linolenate into the liver lipids of groups 2 and 4, and groups 3 and 5 was observed respectively at 1 and 3 hr after the intravenous injection. The specific activity of arachidonate from both of the labeled acids in phospholipid and triglyceride was apparently lower in groups 2 and 3 than in groups 4 and 5, respectively. The distribution rate of radioactivities in tetraenoic acids was also low in groups 2 and 3 among the fatty acids of phospholipid and triglyceride. The results indicated impairment of conversion of homo-γ-linolenate into arachidonate in the cholesterol-fed rats.

INTRODUCTION

Cholesterol accumulation in the liver of rats given cholesterol and cholic acid was reduced by a supplement of linoleate or arachidonate (1), and by a supplement of linoleate to a fat free diet after withdrawal of cholesterol and cholic acid accelerated the regression of cholesterol ester and triglyceride in the liver, compared with a fat free diet and that supplemented with palmitate or oleate (2). The hypocholesterolemic effect of polyunsaturated fatty acid is well known and it was reported that the higher the iodine value of an oil or fat, the lower the effective minimum dose in humans and rats (3,4). Furthermore, it was shown that arachidonate, and eicosapentaenoate and docosahexaenoate exhibited stronger hypocholesterolemic effects than linoleate and α-linolenate, and lowered myocardial lipid levels in rats, even when the iodine value was identical among the diet fats (5). Morin supposed that the arachidonate incorporated into liver phospholipid promoted mobilization of cholesterol and glycerides from the liver of rats given cholesterol (2,6), and that the rise of homo-γ-linolenate was due to the increase of arachidonate synthesis induced by exogenous cholesterol (7). However, the marked increase of homo-γ-linolenate accompanied with the fall in the percentage of arachidonate in the liver of rats fed on cholesterol (8,9) might also indicate an impaired arachidonate synthesis from homo-γ-linolenate.

The authors examine the influence of administration of linoleate and γ-linolenate on the accumulation of exogenous cholesterol in rat liver and plasma, and also the conversion of those fatty acids into arachidonate in the liver.

MATERIALS AND METHODS

Wister strain male rats, weighing 200 to 260 g, were divided into five groups of seven or eight each. Group 1 was given a diet consisting of 10% vitamin free casein, 57.5% sucrose, 20% lard [Hoei Pharmaceutical Co., the main fatty acids: 1.4% 14:0 (number of carbon atoms; number of double bonds), 22.7% 16:0, 4.1% 16:1, 16.9% 18:0, 47.0% 18:1 and 6.7% 18:2], 2% cholesterol (Nakari Chemicals Co.), 1% cholic acid (Nissui Seiyaku Co.), 0.5% choline chloride, 4% salt mixture, 4% cellulose, and vitamin mixture. One fifth of the 20% lard in the

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TABLE I

Plasma Cholesterol Concentration, Liver Lipid Composition and the Content of Fatty Acids of the Linoleate Family (ω6) in Liver Lipids

	Group	Plasma total cholesterol, mg/dl	Liver lipids contents, mg/g wet liver		Fatty acid composition,[a] %				
					18:2	18:3	20:3	20:4	20:4/20:3
1	Chol	626 ± 109[b]	PL[c]	34.8 ± 8.7	9.3 ± 0.9		3.1 ± 0.1	22.7 ± 1.1	7.3
	Lard		TG	53.4 ± 8.8	8.6 ± 0.6	0.1		0.3	
			CH	47.3 ± 8.3	5.1 ± 0.6			0.2	
2	Chol	374 ± 83	PL	31.2 ± 7.7	12.8 ± 2.1		3.3 ± 0.6	23.3 ± 2.1	7.1
	Linoleate		TG	52.0 ± 11.5	19.1 ± 2.0	0.8 ± 0.2	0.6 ± 0.3	0.8 ± 0.4	
			CH	43.6 ± 3.6	12.1 ± 0.7		0.1	0.5	
4	Control	85 ± 9	PL	35.8 ± 6.4	8.6 ± 1.1		0.9 ± 0.2	30.9 ± 0.9	34.3
	Linoleate		TG	13.5 ± 2.4	21.9 ± 2.1	0.9 ± 0.2	0.3	2.9 ± 0.5	
			CH	4.3 ± 1.0	14.9 ± 1.3			11.1 ± 2.5	
3	Chol	249 ± 51	PL	29.8 ± 3.3	8.3 ± 0.8		5.9 ± 1.7	25.7 ± 1.7	4.4
	γ-Linolenate		TG	51.8 ± 21.9	16.8 ± 0.7	3.6 ± 0.8	3.9 ± 2.0	2.3 ± 0.9	0.6
			CH	36.7 ± 3.1	10.4 ± 0.6	0.4	1.5 ± 0.6	1.0 ± 0.2	
5	Control	103 ± 14	PL	32.1 ± 6.6	5.4 ± 0.6		1.7 ± 0.6	33.0 ± 0.8	19.4
	γ-Linolenate		TG	11.3 ± 4.1	18.9 ± 0.6	2.2 ± 0.5	2.0 0.7	8.8 ± 2.0	4.4
			CH	4.9 ± 0.9	11.3 ± 1.4	1.2 ± 0.3	0.9 ± 0.8	11.4 ± 4.1	

[a]Percentage in total fatty acids of each lipid fraction.

[b]Mean ± s.d

[c]Abbreviations: PL, total phospholipid; TG, triglyceride; CH, total cholesterol for liver lipid contents and cholesterol ester for fatty acid composition.

diet was replaced by methyl linoleate (94.5% linoleate and 5.5% 18:1) in group 2 and by methyl linoleate plus methyl γ-linolenate (50.3% linoleate, 47.0% γ-linolenate and 2.7% 18:1, obtained from Ono Pharmaceutical Co.) in group 3. Groups 4 and 5 were given diets similar to those for groups 2 and 3, respectively, but containing no cholesterol and cholic acid. After four weeks on the diets, the rats were anesthetized with ether after overnight fasting, and blood was drawn from the abdominal aorta. The liver was washed with physiological saline through the portal vein and removed. At 1 hr and 3 hr before killing them, the rats received 0.5 ml of an albumin solution containing labeled linoleate in groups 2 and 4, and labeled γ-linolenate in groups 3 and 5, by injection into the tail vein.

A portion of the plasma in each animal was used for examination of lipid concentrations and the remainder (1 ml/rat) was pooled in each group to be extracted in chloroform-methanol (2:1). Two grams of each liver was immediately homogenized in methanol for extraction in chloroform-methanl (2:1). The phospholipid, triglyceride and total cholesterol contents were measured with a small part of the extract. The extracts from the plasma and liver obtained by Folch's method (10) were fractionated into major lipid classes by silicic acid thin layer chromatography (TLC) developed with petroleum ether-diethyl ether-glacial acetic acid (85:14:1) as solvents. The fractions of cholesterol ester, triglyceride, free fatty acid and free cholesterol plus diglyceride were eluted with diethyl ether, and the phospholipid fraction with methanol.

Radioactivity in each fraction of the liver lipids was counted and the remainder was transmethylated with dry methanol containing 5% HCl for 3 hr at 65 C. The methyl esters of fatty acids were separated by silicic acid TLC using toluene as the developing solvent. Aliquots were taken for analyses by gas liquid chromatography (GLC) and the remainder was fractionated according to the number of double bonds on $AgNO_3$-impregnated silicic acid TLC (11). The plasma lipid fractions were transmethylated and analyzed by GLC.

Argentation TLC was carried out on a 23 X 8 cm glass plate coated with Silica Gel G (E. Merk Ltd.) impregnated with 25% $AgNO_3$, 300 μm in thickness, with chloroform-methanol (95:5) as solvent. Fractions were made visible under the UV lamp by spraying with dichlorofluorescein. To each fraction scraped off, diethyl ether and the same volume of 15% methanol were added, and the mixture was shaken vigorously. This extraction was repeated once more. Radioactivity of each fraction was counted after the fatty acid fractionations were tested by GLC on all samples. The saturated and monoenoic, dienoic, trienoic and tetraenoic fatty acids were clearly separated from each other, but a small part of arachidonate was sometimes found in the fraction of pentaenoic and hexaenoic acid. The recovery rate of methylated fatty acids through argentation TLC was about 95%, and no difference was found between the recovery rates of saturated acids and those of unsaturated ones. These procedures were carried out under nitrogen gas as much as possible, especially when the solvents were evaporated and the samples were preserved.

GLC was carried out with Yanagimoto Gas Chromatograph 550F, equipped with a 220 cm glass column packed with 20% diethylene glycol-succinate on celite and a hydrogen flame detector. Analyses were performed at a column temperature of 206 C, an injection temperature of 240 C and a nitrogen flow of 20 ml/min. The peak areas were calculated by triangulation. Technical error (coefficient of variation) for the fatty acid composition was less than 5% for major components (5% or more of total fatty acids) and less than 10% for minor components (from 1% to 5% of total fatty acids).

Fatty acids were identified by their retention time on GLC and fractionation by argentation TLC. Fatty acids used as standards for identification in both methods were as follows: 18:0 (99% pure) 20:0 (96%), 22:0 (95%), 18:1ω9 (number of carbon atoms from the methyl end to the first double bond), 18:2ω6 (99%), 18:3ω6 (95%), 18:3ω3 (95%), 20:3ω6 (96%) and 20:4ω6 (95%) (Nakarai Chemicals Co., Ono Pharmaceutical Co. and Mann Research Laboratories). In the liver and plasma lipids of rats, the following acids were found: 14:0, 16:0, 16:1, 18:0, 18:1, 18:2, 18:3ω6, 18;3ω3, 20:0, 20:1, 20:2, 20:3ω9, 20:3ω6, 20:4ω6, 20:5ω3, 22:0, 22:4ω6, 22:5ω6, 22:5ω3, 22:6ω3, 24:0 and 24:1ω9.

The triglyceride (12), phospholipid (13) and total cholesterol (14) contents were measured according to the previous reports.

The linoleate-1-^{14}C (51 mCi/mM) and methyl γ-linolenate-1-^{14}C (20mCi/mM) were obtained from Daiichi Chemicals Co. Ltd. The purity of both acids proved to be more than 99% by radio-GLC (Shimazu RID-2A, with the column packed with 20% diethylene glycol-succinate on celite). Unlabeled linoleate (99%) was added to the labeled linoleate and the mixture was dissolved in 15% bovine albumin solution (tris-buffer PH 7.4). The labeled methyl γ-linolenate was mixed with the unlabeled methyl γ-linolenate which had been purified by

TABLE II

The Specific Activities (cpm/mg) of 1-^{14}C-Linoleate and 1-^{14}C-γ-Linolenate in Liver Lipids and of the Linoleate and Arachidonate in Liver Phospholipid and Triglyceride

	Group	Time,[a] hr	PL	TG	CH[c]	PL[b,d]		TG[b,d]	
						Linoleate	Arachidonate	Linoleate	Arachidonate
2	Chol	1	1579 ± 353[e]	1257 ± 446	296 ± 92	11196 ± 612	595 ± 49	5204 ± 2348	f
	Linoleate	3	814 ± 236	1351 ± 213	333 ± 59	5919 ± 1053	709 ± 130	6020 ± 2217	
4	Control	1	1867 ± 189	5407 ± 2463	418 ± 97	16109 ± 2457	1866 ± 113	18049 ± 6804	
	Linoleate	3	1078 ± 332	2736 ± 701	385 ± 145	10006 ± 2009	1132 ± 792	8011 ± 2285	
3	Chol	1	1084 ± 507	1120 ± 572	80 ± 36		800 ± 170		1501 ± 376
	γ-Linolenate	3	566 ± 59	1219 ± 428	85 ± 22		701 ± 250		1483 ± 405
5	Control	1	1870 ± 845	5276 ± 2055	244 ± 114		4824 ± 2492		10182 ± 2260
	γ-Kubikebate	3	1196 ± 135	1600 ± 199	157 ± 41		3239 ± 547		4585 ± 359

[a]After intravenous injection of 1-^{14}C-linoleate and 1-^{14}C-γ-linolenate.

[b]Abbreviations; PL, total phospholipids; TG, triglyceride.

[c]For total cholesterol in the liver.

[d]The radioactivities of dienoic and tetraenoic acids, fractionated on argentation TLC, were assumed to be equivalent to those of linoleate and arachidonate, respectively.

[e]Mean ± s.d

[f]Not calculated because of a small percentage of arachidonate.

TABLE III

Distribution of the Radioactivity From 1-^{14}C-Linoleate and 1-^{14}C-γ-Linolenate in the Fatty Acids of Liver Phospholipid

	Group	Time,[a] hr	Number of double bonds in fatty acids, %				
			0+1	2	3	4	5-
2	Chol Linoleate	1	24.6	61.7	8.2	4.7	0.8
		1	29.4	55.0	8.5	6.7	0.4
		1	19.7	68.4	5.7	5.1	1.1
		3	15.2	60.8	8.4	14.3	1.3
		3	19.9	57.0	6.9	13.6	2.6
4	Control Linoleate	1	18.5	55.1	6.2	18.9	1.3
		1	16.4	51.7	4.2	25.8	1.9
		3	16.1	63.8	3.3	16.5	0.3
		3	11.0	58.7	5.5	18.1	6.7[b]
		3	10.1	48.9	7.0	31.5	2.5
			0+1+2				
3	Chol γ-Linolenate	1	7.1		77.8	14.9	0.2
		1	8.5		72.5	18.7	0.3
		1	4.3		71.4	19.8	4.5[b]
		3	6.2		55.7	36.3	1.8
		3	7.0		76.2	16.3	0.5
		3	7.3		78.7	13.8	0.2
		3	8.0		63.1	28.2	0.7
5	Control	1	3.3		36.9	59.0	0.8
		1	4.5		33.4	56.6	5.5[b]
		1	8.4		42.6	47.8	1.2
		3	4.4		28.3	65.8	1.5
		3	3.0		29.8	66.6	0.6
		3	3.8		32.1	63.1	1.0
		3	7.6		28.4	61.1	2.9

[a]After intravenous injection of 1-^{14}C-linoleate and 1-^{14}C-γ-linolenate.
[b]Mixed with a small portion of tetraenoic acid on argentation TLC.

argentation TLC. The mixture of methyl γ-linolenate was hydrolyzed under nitrogen gas in ethanol with NaOH at 55 C for 30 min. After distilled water was added, extraction with petroleum ether (for the remaining methyl γ-linolenate) was repeated three times. Then the phase of ethanol in water was acidified with HCl and was extracted with petroleum ether (for the free γ-linolenate). About 90% of the γ-linolenate was recovered in a free form and was dissolved in an albumin solution. To each rat was injected 0.5 ml of the albumin solution containing 7 μCi of 1-^{14}C-linoleate or 1-^{14}C-γ-linolenate with 1 mg of the respective fatty acid. The lipid samples for counting of radioactivity were eluted in 3 ml of methanol and 7 ml of toluene containing 5 g of 2,5-diphenyl oxazole (DPO, Wako Chemicals Co.) and 100 mg of 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene (dimethyl POPOP, Packard Instrumental Co. Ltd.) per liter. Radioactivity was examined by the liquid scintillation counter, LSC601 (ALOKA Co. Ltd.). The probabilities that apparent differences in the date were due to chance were calculated by the t test.

RESULTS

Lipid Concentration of the Plasma and the Liver

A large white-colored liver with accumulation of triglyceride and cholesterol was found in all of the rats given cholesterol (Table I). Group 3 had less deposit of cholesterol in the liver than the other cholesterol-fed groups ($P<0.01$). The cholesterol-fed groups also showed elevated concentrations of plasma cholesterol in comparison with control groups, and among the cholesterol-fed groups, the more unsaturated the fatty acids in the diet fats were, the lower the cholesterol concentrations were (group 1>2: $P<0.001$, group 2>3: $P<0.005$).

Fatty Acid Composition of Liver Lipids

In the total phospholipids, homo-γ-linolenate and arachidonate represented higher percentages in the cholesterol-fed group given γ-linolenate (group 3) than in that given linoleate (group 2) (20:3, $P<0.005$ and 20:4, $P<0.05$), and the cholesterol-fed groups had higher percentages of linoleate and homo-γ-linolenate, and a lower percentage of arachidonate than

TABLE IV

Incorporation of 1-^{14}C-Linoleate and 1-^{14}C-γ-Linolenate Into the Fatty Acids of Triglycerides in Rat Liver

	Group	Time,[a] hr	Number of double bonds in fatty acid, %			
			0+1	2	3	4-
2	Chol	1	22.8[b]	67.9	8.3	1.0
	Linoleate	3	15.9	77.7	4.7	1.8
4	Control	1	21.3	70.9	5.6	2.2
	Linoleate	3	19.1	73.1	5.3	2.5
3	Chol	1	1.9	5.3	89.8	3.0
	γ-Linolenate	3	2.4	4.6	89.7	3.3
5	Control	1	1.9	4.4	77.4	16.3
	γ-Linolenate	3	3.4	7.7	63.3	25.6

[a]After intravenous injection of 1-^{14}C-linoleate and 1-^{14}C-γ-linolenate.
[b]Average of three to four rats.

the control groups (Table I). There was no distinct difference among all groups in the percentages of ω3 polyenoic acids such as 20:5, 22:5 and 22:6. In the triglycerides, the percentages of γ-linolenate and homo-γ-linolenate were elevated in the γ-linolenate-fed groups, especially in group 3; a high percentage of arachidonate was found only in group 5. In the liver cholesterol ester, the cholesterol-fed groups showed a markedly decreased percentage of arachidonate, compared with control groups.

Fatty Acid Composition of Plasma Lipids

The fatty acid composition of total phospholipids in the plasma closely agreed with that in the liver in each group. The plasma triglycerides showed increased percentages of arachidonate (group 2 7.7%, 3 8.6%, 4 7.2% and 5 18.3%) at the expense of 18:1, compared with the liver triglycerides. Although arachidonate represented a smaller percentage in cholesterol-fed groups than in control groups, the percentages were significantly different from one another (group 1, 3.7%<2, 6.1%<3,10.9%).

Specific Activities of Some Lipid Fractions and Fatty Acids in the Liver

The specific activity of phospholipid and triglyceride at 1 hr after injection was higher than at 3 hr in control groups, whereas in cholesterol-fed groups, the specific activity of triglyceride showed no difference between 1 hr and 3 hr. This suggested that the turnover rate of triglyceride in the latter groups may be slow. The specific activity of cholesterol in Table II was calculated for total cholesterol, since the cholesterol ester content was not measured. In the labeled γ-linolenate-injected groups, the specific activity of cholesterol was higher in the control group than in the cholesterol-fed group.

In phospholipid and triglyceride of the liver, the specific activities of linoleate and arachidonate were calculated, assuming that the radioactivities of the fractions of dienoic and tetraenoic acids were equivalent to those of linoleate and arachidonate, respectively. The specific activity of arachidonate of the control group was markedly higher than that of the cholesterol-fed group in ^{14}C-γ-linolenate-injected rats, and slightly higher in ^{14}C-linoleate-jected rats. It fell from 1 hr to 3 hr after injection in the control groups both in phospholipid and triglyceride, but did not change in the cholesterol-fed groups. The specific activity of linoleate was also lower in the cholesterol-fed group than in the control group, but the difference was smaller than in that of arachidonate.

Distribution of the Radioactivity of 1-^{14}C-Linoleate and 1-^{14}C-γ-Linolenate to the Fatty Acids Fractionated According to the Degree of Unsaturation in Liver Phospholipid and Triglyceride

In phospholipid fatty acids, the rate of incorporation of radioactivity of ^{14}C-linoleate in tetraenoic acids was slightly lower in the cholesterol-fed group than in the control, whereas that in trienoic acids showed no significant difference between the two groups. A more apparent difference between the cholesterol-fed group and the control was observed in the distribution of radioactivity of ^{14}C-γ-linolenate. In the control group, about 50% of the radioactivity was distributed to tetraenoic acids at 1 hr and 60% at 3 hr, whereas in the cholesterol-fed group, less than 36% was distributed even at 3 hr after injection (Table III).

Similar differences were found in triglyceride fatty acids between the cholesterol-fed groups and the controls. In all groups, the rate of incorporation of radioactivity in tetraenoic acids was far lower in triglyceride than in phospholipid (Table IV).

DISCUSSION

The hypocholesterolemic effect of γ-linolenate was stronger than that of linoleate, and hepatic cholesterol accumulation was less in γ-linolenate-fed rats. Since γ-linolenate may be easily converted to arachidonate in vivo (15), it is possible that the hypocholesterolemic effect was due to arachidonate synthesized from γ-linolenate in the liver. In fact, arachidonate contents of the liver and plasma increased in the γ-linolenate-fed rats, compared with those in the linoleate-fed rats. Arachidonate is reported to have an important function for the metabolism of excess cholesterol in rat liver (2,16) and a strong hypocholesterolemic effect (3,5). Some investigators have reported that cholesteryl arachidonate in rat plasma was hydrolyzed faster than other cholesterol esters (17) and would have a rapid turnover rate (18).

However, it is doubtful that arachidonate was synthesized in cholesterol-fed rats as smoothly as in control rats. Altough the amount of arachidonate in the liver calculated from the date in Table I was somewhat larger in cholesterol-fed rats than in control rats, the increase of γ-linolenate and homo-γ-linolenate in cholesterol-fed rats was more remarkable. The ratio of arachidonate to homo-γ-linolenate was distinctly smaller in cholesterol-fed rats than in control rats. Even if the reduction of relative amounts of arachidonate was due to an increase of total liver lipids, the turnover rates of liver lipids or fatty acids should also be taken into consideration in cholesterol-fed rats.

The ratio of arachidonate to homo-γ-linolenate in liver phospholipid and triglyceride was lowered by γ-linolenate feeding in both the cholesterol-fed group and the control. Similar results have been reported previously (19). The synthesis of arachidonate from linoleate might be inhibited by the supplement of γ-linolenate. The experiment in vitro with rat liver microsomes by Marcel et al. (20) suggests that γ-linolenate is more easily converted into homo-γ-linolenate than is homo-γ-linolenate into arachidonate. However, the latter conversion was strongly enhanced by 18:2, 18:3 and 20:2 of the linoleate family.

If exogenous cholesterol accelerates arachidonate synthesis, the major part of the increased arachidonate must be transported to the plasma cholesterol ester fraction via phospholipid (6,21,22), since there is very little arachidonate in the liver cholesterol ester. However, the specific activity of arachidonate in liver phospholipid after the injection of 1-^{14}C-linoleate or 1-^{14}C-γ-linolenate was distinctly lower in the cholesterol-fed group than in the control. It was also shown that the incorporation of the radioactivity into tetraenoic fatty acids in phospholipids and triglycerides was lower in the cholesterol-fed group than in the control, and that there was no significant difference between the two groups in the incorporation of the radioactivity of ^{14}C-linoleate into trienoic acids in phospholipid and triglyceride. These results suggest that the conversion of homo-γ-linolenate into arachidonate was impaired in the liver of cholesterol-fed rats and that the decreased arachidonate synthesis might promote the cholesterol accumulation in the liver and plasma. Since, in this experiment, radioactivity was examined only at 1 hr and 3 hr after the injection of labeled fatty acids, it is possible that the conversion of homo-γ-linolenate into arachidonate was delayed in the cholesterol-fed group.

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Lipolysis in Castor Seeds: A Reinvestigation of the Neutral Lipase

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ABSTRACT

Attempts to study the neutral lipase reported in germinating castor seeds and to compare this enzyme to the acid lipase of dormant seeds were unsuccessful because of the inability to repeat the work described earlier (1). Activity of the proposed neutral lipase could not be detected, nor could appearance of the enzyme be hastened or affected by treating the germinating seeds with gibberellic acid or Actinomycin-D. Possible explanations for the discrepancy between these findings and the early report are presented.

INTRODUCTION

A lipase (E.C. 3.1.1.3) or the dormant castor seed, *Ricinus communis L.*, has been studied extensively and its properties and pH optimum are well established (1-4). During subsequent investigations on the enzyme, a lipid cofactor was isolated (5,6). The term "acid lipase" for the dormant seed enzyme was then introduced to distinguish it from the neutral lipase reported by Yamada (1). He mentioned two lipases in castor seeds, one in dormant seed with optimum activity at acid pH (the acid lipase) and another which appeared after germination with a pH optimum at 6.8.

St. Angelo and Altschul (7) showed that acid lipase activity of dormant castor seeds increased up to 2-3 days after germination and continued for seven days. At this point, most or all of the storage oil in the endosperm was depleted (7,8). Since the acid lipase is associated with the spherosomes which contain the oil in the endosperm (9), the concept of a second lipase, with apparently different properties than the original enzyme and appearing at a stage of germination when the oil is rapidly depleting, seemed unusual since the acid lipase is still quite active.

Investigations were begun about 1961 to compare the two lipases and to relate lipolysis in dormant seeds to that in germinating seeds. Experiments were designed to determine if the two enzymes were one and the same, or if the neutral lipase was newly synthesized during germination. At intervals through these years and with each new supply of seeds received, attempts have been made to repeat Yamada's findings of a neutral lipase. Since lipolysis could not be detected at pH 6.8-7.2 under his conditions, the procedure was modified to hasten appearance of the neutral lipase by gibberellic acid (GA) stimulation, as applied to lipase synthesis in cottonseed (10).

The data, necessarily condensed, are from experiments of 1967-1969, during which time the techniques used were considerably improved. However, they typify the data collected over the entire period during which these attempts to locate the proposed neutral lipase were made.

EXPERIMENTAL PROCEDURES

In early investigations (1961-1964), Cimarron variety of castor seeds were used. After this, Baker 296 variety was substituted and is

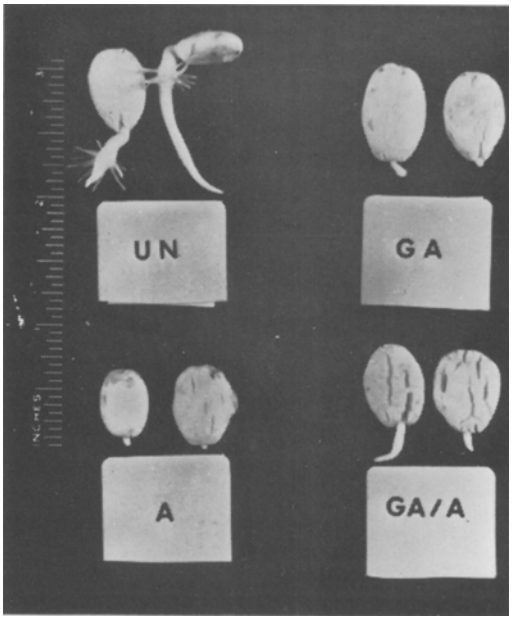

FIG. 1. Effect of GA and Actinomycin-D on visible growth of castor seeds. Seeds germinated in Petri dishes as described in Experimental Procedures. Un, untreated; GA, GA-treated; A, Actinomycin-treated; GA/A, GA and Actinomycin-treated.

[1]So. Utiliz. Res. Dev. Div., ARS, USDA.

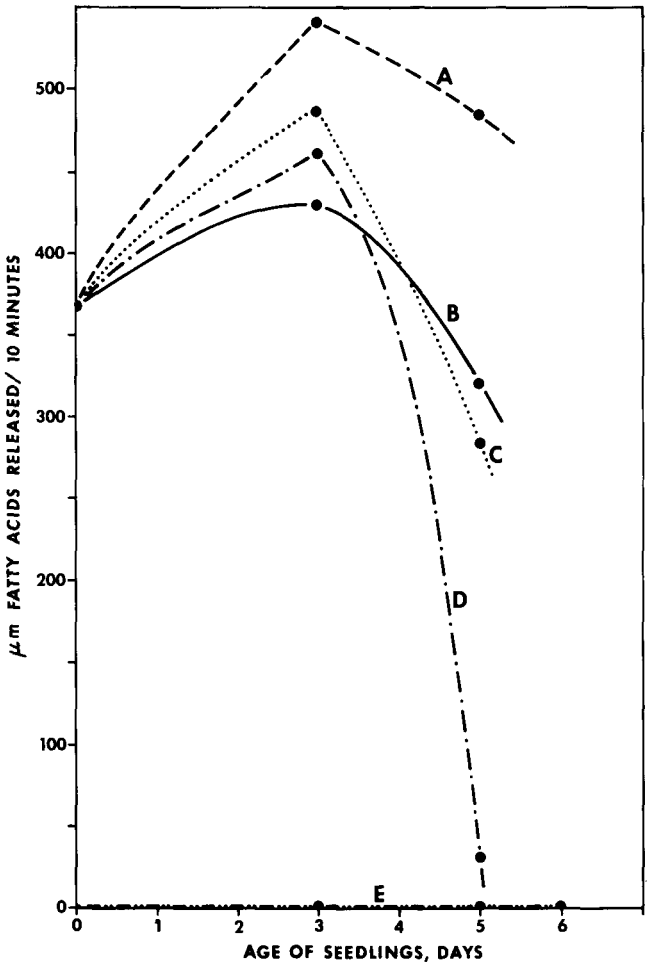

FIG. 2. Effect of GA and Actinomycin-D on lipase activities in castor seeds. Conditions described in Experimental Procedures; cottonseed oil served as the substrate in the experiments shown. Curves for acid lipase activity: A, Actinomycin-treated; B, untreated; C, GA and Actinomycin-treated; D, GA-treated; E, neutral lipase activity for all treatments.

the seed used in the studies reported here. GA was purchased from Eastman Chemicals Co.; substrates were commercial sources of refined cottonseed or corn oil. Spergon and Sterosan were commercial microbial inhibitors; all other chemical reagents were purchased from local suppliers.

Preparation of Neutral Lipase Extract

The procedure of Yamada (1) was used. This consisted of homogenizing the endosperms or decotylated embryos of germinated seeds in 90% glycerol (pH 7.6), centrifuging at 400 x *g* for 4-5 min to remove cell debris, and homogenizing the supernatant which then served as the enzyme source. One ml of homogenate was equivalent to 100 mg fresh tissue.

Assays for Neutral Lipase Activity

Yamada's method (1) of titrating the fatty acids released by the homogenate in 10 min was followed exactly in some experiments and modified in others. He titrated the fatty acids released from a Tween-60 substrate with NaOH using a phenyl red indicator. This procedure was tried in the present studies. In addition, since some authors (11-13) either reject the use of Tweens as lipase substrates or refer to the enzyme hydrolyzing Tweens as a "Tweenase" rather than a lipase, the Tween substrate was replaced by refined cottonseed or corn oils. Enzyme and substrate were homogenized in a Potter-Elvehjem tube, then the fatty acids released during the 10 min reaction time were titrated to a preset endpoint (pH 8.5) in the

Radiometer pH-Stat as reported for acid lipase activity (4), rather than to a visual endpoint.

Preparation of Seeds for Germination

The seedcoat of the castor seed is quite hard. Therefore, some experiments were conducted on intact kernels carefully dissected by hand to remove the seedcoat, to obtain more even imbibition of water during germination. Intact seeds were lightly dusted with Spergon or Sterosan prior to wetting with water and germinated at 25 C. Free kernels were sterilized by immersion in ice cold 1% sodium hypochlorite for 15 min, then rinsed twice in sterile, cold distilled water for 10 min periods. Germination of kernels took place in sterile Erlenmeyer flasks aerated by mild wrist action shaker motion at 23 C or between moist filter paper in sterile Petri dishes at 25 C. Intact seeds were germinated in moist Vermiculite.

GA and Actinomycin-D Treatments

These were conducted on intact kernels in Erlenmeyer flasks and Petri dishes. GA (10^{-5} M) and Actinomycin-D (50 μg/ml) were sterilized by filtration through bacterial filters and added to the incubation vessels. Concentrations used were the same as those employed for the cottonseed lipase (10).

RESULTS AND DISCUSSION

Assays for Neutral Lipase Activity

All tests reported were conducted on homogenates of endosperms only, from seeds germinated with or without seedcoats, as done in the original report (1). In all cases, seeds and kernels were surface sterilized before germination. The Tween-60 solution of Yamada (1), corn oil and cottonseed oil were employed as substrates for reaction times of 10 to 60 min. In one series, threefold increases in the amount of enzyme homogenate were tested with corn oil substrate for a 60 min period without obtaining measurable lipolysis at pH 6.8-7.2. While acid lipase activity at pH 4.2 of these same homogenates varied in individual tests from 176 to 429 μ moles fatty acids released per 10 min reaction period, in no case was neutral lipase activity detected.

St. Angelo and Altschul (7) examined the free fatty acid pools in germinating castor seeds, peanuts and cottonseeds. Lipase activity in dormant seeds was found only in castor beans but in all three germinating seeds; lipase activity was optimal at acid pH. Since lipolysis was detected at neutral pH, they suggested that such activity might be due to microbial action.

Effects of GA and Actinomycin on Lipolysis

If microbial growth were the source of this neutral lipase activity, it seemed worthwhile to hasten appearance of the seed lipase, if possible, before appreciable microbial growth occurred. Therefore, GA was applied to sterile kernels minus seedcoats. As additional controls, some kernels were treated simultaneously with Actinomycin-D and with Actinomycin-D alone to block synthesis of any new lipase which might be formed during germination.

Visual examination of the smallest and the largest kernels removed from each group after four days germination showed some effects of the applied treatments (Fig. 1). All seeds imbibed water but only untreated seeds showed prominent root growth. Endosperms of GA-treated seeds swelled the most of the three groups at this stage, suggesting that the greatest amount of physiological activity might be present in these. Actinomycin-treated seeds were the smallest. While visible growth of the seedlings seems to be inhibited by the treatments, physiological changes in the germinating endosperms were apparently high. As seen in Figure 2, disappearance of acid lipase activity in GA-treated seeds (D) was accelerated over the untreated control (B). The increased activity of acid lipase activity in Actinomycin-treated endosperms (A) may be due to blocked synthesis of autolytic enzymes which would normally remove the lipase. The combined effects of GA and Actinomycin (curve C) were added as an additional, treated control. The results appear to fall between each individual treatment, suggesting a possible mutual neutralization of the effects by each additive. The important fact derived from these series, however, was the complete absence of neutral lipase activity in both Cimarron and Baker 296 varieties of castor seeds under all conditions of germination and testing, whether seeds were germinated in aerating Erlenmeyer flasks, still flasks, or in Petri dishes.

The original goal of these investigations was to determine if the neutral lipase was a zymogen which was activated upon germination or was a result of de novo synthesis. Our inability to detect neutral lipase activity under all conditions tested and on several lots of seeds suggested that the neutral lipase activity reported earlier (1), if present at all, might have been derived from a source other than the seed.

Microbial lipases have been studied by numerous groups. Dirks et al. (14) compared lipases of wheat germ and *Aspergillus* and suggested that fungal lipases might be responsible for the observed fat acidity in cereal grains; both had pH optima at 7.0-7.3. Ramakrishnan

and Banerjee (15-17) found that fungal lipases grown on oilseeds were more active than the endogenous seed lipases. They compared lipases from castor seed and two strains of *Aspergillus* which had been isolated from castor seeds and showed that the fungal lipases were most active at pH 6.2-7.2 (16). Activity of the fungal lipase increased with age of the seedling to a certain point before decreasing, while that of the lipase from these infested seeds decreased steadily. A lipolytic esterase in *Staphylococcus* (18) was shown to hydrolyze Tweens as well as glycerides, but in our studies neither Tweens nor glycerides were hydrolyzed at neutral pH.

While both an acid and a neutral lipase have been reported in fir seeds (19), the results of our extensive investigations indicate that the Cimarron and Baker 296 varieties of castor seed do not contain a neutral lipase. Until further evidence for the presence of this enzyme is forthcoming, it may be advisable to refer to this as a proposed neutral lipase rather than as an accepted fact, as has been done in at least one book (20).

ACKNOWLEDGMENTS

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The Relationship of Milk Phospholipids to Membranes of the Secretory Cell

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ABSTRACT

Forty-two per cent of the lipid phosphorus in milk was found in skim milk lipoprotein; the other 58% occurs in the milk fat globule membrane (MFGM). Investigation of these two sources of lipid phosphorus revealed that they involve the same individual phospholipids, in essentially the same proportions with similar fatty acid compositions. Both contain sphingomyelin and cerebrosides in levels characteristic of those found in plasma membranes. Other points of resemblance between MFGM and skim milk lipoprotein, have been shown previously. Infusion of (^{14}C) palmitate into the mammary gland of a lactating goat produced more extensive labeling of all the phospholipid classes in the skim milk lipoproteins than in those in the MFGM during the following 24 hr. When (^{14}C) palmitate was infused into the jugular vein of a lactating goat, a precusor-product-type relationship was observed between specific activities of the skim milk and MFGM polar lipids. These results render the MFGM an unlikely origin of the skim milk lipoprotein. Other possible sources of this latter lipoprotein are Golgi vesicle membranes or plasma membrane of the lactating cell.

INTRODUCTION

Consideration of morphological and biochemical evidence (1-4) leaves little doubt that the milk fat globule membrane (MFGM) originates directly from plasma membrane which envelopes the globule at secretion (2). A question is then raised regarding lipoprotein in the skim milk phase. This material accounts for one third to one half of the lipid phosphorus of milk (5-7) and it bears considerable likeness to MFGM. The proportions of the various phospholipids in the two and the fatty acid compositions of the phospholipids are similar (7,8). Similar enzymatic activities have also been demonstrated in the two sources (9-11). The skim milk lipoprotein may simply be material that is shed from the MFGM, but radioisotope tracer experiments indicate that this explanation is inadequate.

MATERIALS AND METHODS

In the analyses of phospholipids in fat globules and skim milk, freshly drawn milk samples were obtained from cows (Holstein) and goats (Nubian). They were divided into fat globules and skim milk by centrifugation at 30,000 to 35,000 x *g* for 1 hr at 5 C. Lipids were recovered from these samples by Roese-Gottlieb extraction (12). Solvents were removed by rotary evaporation under reduced pressure at room temperature. Polar lipids were separated from crude lipid extracts by silicic acid column chromatography (13). The polar lipids, eluted with methanol, were recovered by evaporation as above and immediately redissolved in an accurately measured volume of chloroform. These lipids were separated by two dimensional thin layer chromatography (TLC) (14). Levels of phospholipids were determined by phosphorus analysis of total lipid fractions or of areas scraped from thin layer plates (15). Identities of phospholipids separated by this procedure have been established (2,14,16). Fatty acid compositions of phospholipids separated by TLC were determined by gas chromatography of methyl esters as described previously (2,17).

To explore the relationship between the lipoproteins of skim milk and the MFGM, we conducted two tracer experiments with Na-1-(^{14}C) palmitate (Applied Science, State College, Pa.). In the first experiment, a goat in mid lactation was completely milked and the tracer (50 μC, 1 μM), in 5 ml of water, was infused into the mammary gland via a teat canal. Milk from that side of the gland was collected at hourly intervals for the first 6 hr, then at 9, 12 and 24 hr postinfusion. Fat globules were separated from skim milk in these milk samples by centrifugation at 6 200 x *g* for 6 min at -2 C. The plastic tubes (50 ml) of centrifuged milk were completely frozen at -30 C and then sawed just below the compacted layers of milk fat globules. In a second infusion experiment, 100 μC of Na-1-(^{14}C) palmitate (57 μC/μM, Amersham/Searle, Des Plaines, Ill.) was bound to 20 mg of bovine serum albumin

in 5 ml of water and injected into the jugular vein of a completely milked lactating goat. Milk samples were collected at 2 hr intervals for the first 12 hr, then at 24 hr post infusion. These milk samples were separated into MFGM and skim milk fractions by published methods (2,7). Isolation of MFGM was accomplished by freezing centrifugally compacted layers of fat globules at 4 C for 24 hr, thawing and melting the material at 40 C, diluting with several volumes of water and sedimenting the membrane material into a pellet at 36,000 x *g* for 2 hr. Extraction of the lipids, isolation and separation of phospholipids and determination of lipid phosphorus in these fractions were accomplished by the methods cited here. Specific activities of phospholipids were calculated from radioactivities and phosphorus contents of total polar lipid fractions or of the appropriate areas scraped from two dimensional thin layer chromatograms. Specific activities per microgram P were converted to a phospholipid basis by the factor, X 40.

RESULTS

Analysis of milks from four individual cows revealed an average of 42.4% of the total lipid phosphorus in the skim milk (range of 39.9% to 43.5%). The milk of an individual goat yielded 42.0% of the lipid phosphorus in the skim milk. The remainder of the lipid phosphorus is contained in the MFGM; none was dissolved in the fat (5,7). The same phospholipid classes were associated with fat globules and in skim milk. Only small variations were observed in the distribution of the individual phospholipids between skim milk and globules of a given milk and the average values for the four samples show close agreement for levels of the individual components in the comparison between skim milk and globules (Fig. 1). Some minor variation in phospholipids at the two sites would be expected on the basis that secretion involves the addition of plasma membrane phospholipids to those that may preexist on the fat droplet within the cell (2,3). Mono- and dihexose cerebrosides, constituents characteristic of plasma membranes and MFGM (2,17), were present in both globules and skim milk from all samples. Comparative fatty acid analyses (not shown) for phosphatidyl inositol, phosphatidyl ethanolamine and phosphatidyl choline from globules and skim milk confirmed earlier findings regarding the similarities in compositions for the two sites (7,8).

Data from the experiments comparing specific activities of phospholipids in skim milk with those of fat globules after infusion of Na-

TABLE I

Radioactivity Incorporation Into the Phospholipids of Skim Milk and of Milk Fat Globule Membrane at Various Intervals of Milking After Infusion of 50 μC of Na-1-(^{14}C) Palmitate Into the Udder of a Lactating Goat

Milking Interval, hr	Specific activity, cpm/mg									
	Phosphatidyl ethanolamine		Phosphatidyl serine		Phosphatidyl choline		Phosphatidyl inositol		Sphingomyelin	
	G[a]	S[a]	G	S	G	S	G	S	G	S
1	428	1796	0	428	1752	16096	640	6964	196	692
2	76	448	0	100	160	2964	80	720	172	800
3	36	248	40	269	172	1768	0	648	60	900
4	32	128	24	32	80	792	0	612	60	448
5	10	244	0	256	92	1054	4	476	84	564
6	12	212	0	152	116	1084	0	120	80	584
9	20	b	16	b	84	b	20	b	72	b
12	16	56	0	280	68	268	0	240	60	352
24	12	28	0	80	40	116	0	60	68	368

[a]Abbreviations: G, globules; S, skim milk.
[b]Samples accidently lost.

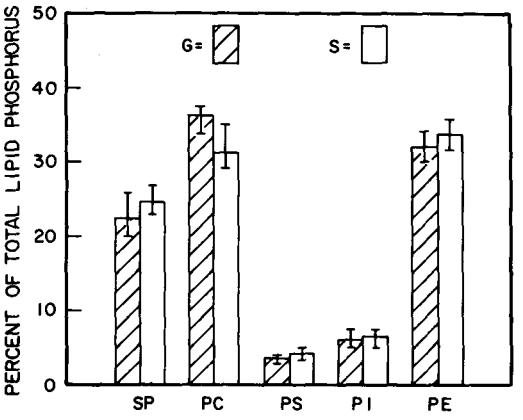

FIG. 1. Distribution of phospholipids as per cent of total lipid phosphorus in fat globules (G) and skim milk (S). The averages and ranges encountered in four individual milk samples are presented. Abbrevitions: PE, phosphatidyl ethanolamine; PI, phosphatidyl inositol; PS, phosphatidyl serine; PC, phosphatidyl choline; Sp, sphingomyelin.

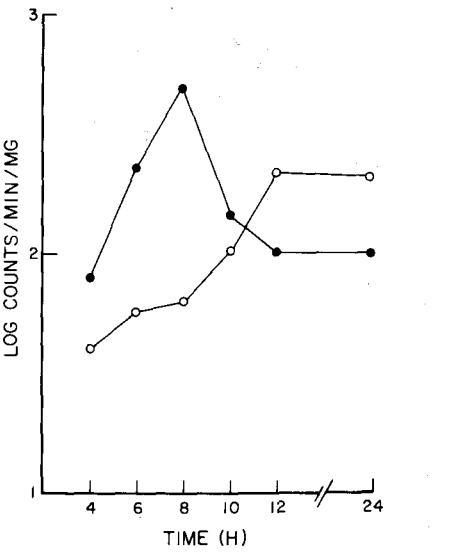

FIG. 2. Changes in specific activity of goat fat globule membrane and skim milk polar lipid fractions after intravenous infusion of 100 μC of sodium-1-(^{14}C) palmitate. ●——●, skim milk; ○——○, fat globule membrane.

1-(^{14}C) palmitate into one side of a goat's udder are given in Table I. These data clearly establish the incorporation of the tracer fatty acid into all of the phospholipids of the skim milk and at substantially higher levels than in those of the globules. Since some slight contamination of the globule layers with skim milk was unavoidable in separating the phases, the levels of radioactivity in the globule phospholipids appears to be low indeed. The total incorporation of radioactivity into ester lipids (mainly triglycerides) of the milk during 24 hr was 8.3% of the total infused activity with 0.25% of this dose being recovered in the phospholipids. While phospholipids of the MFGM were relatively devoid of radioactivity throughout the experiment, the neutral glycerides within the globules contained greater than 90% of the incorporated activity. This is good evidence that the tracer was metabolized by the lactating cell. With the exception of the 1 hr datum for phosphatidyl choline in skim milk (Table I), none of the individual phospholipid specific activities at a given time approached those of the neutral lipids in the fat globules.

The conversion of palmitate to other labeled metabolites was not evaluated in this experiment; however, we have previously observed, under these same conditions, that between 90% and 100% of the activity incorporated into lipids remains as palmitate. Our analyses indicate that the palmitate concentrations in total and individual phospholipids from milk fat globules and skim milk are quite comparable. As shown in Figure 1 there is slightly more phosphatidyl choline in milk fat globules. For the purpose of evaluating activity data in Table I the following concentration of palmitate in the individual phospholipids can be used; phosphatidyl choline 33%, phosphatidyl ethanolamine 14%, sphingomyelin 15%, phosphatidyl inositol 13% and phosphatidyl serine 8%. It can be seen that the labeling pattern (Table I) does not strictly follow the amount of palmitate in the particular phospholipids although phosphatidyl choline has the highest levels of activity, and of palmitate.

Results from the experiment comparing the specific activities of total polar lipid fractions of skim milk and MFGM after injection of Na-1-(^{14}C) palmitate into the circulation are depicted in Figure 2. In contrast to the previous experiment where the isotope was introduced directly into the mammary gland, in this case, labeling of phospholipids was much lower due to slow entry of the administered acid into the mammary gland over a prolonged period. While the skim milk polar lipids were maximally labeled 8 hr postinfusion, the specific activity of MFGM polar lipids did not reach a maximum value until 12 hr postinfusion. In this experiment approximately 3.4% of the total dose administered was recovered in the milk lipids during 24 hr.

DISCUSSION

The findings of this study are in harmony with other findings (2,7-11) showing sub-

stantial resemblance between the lipoproteins of skim milk, MFGM and plasma membrane of the lactating cell. Our data do not support the concept that the skim milk lipoprotein arises by disintegration of MFGM. Although the results of the ^{14}C-tracer experiments pose some difficulties of interpretation, they show clearly that the skim milk phospholipids become labeled more promptly and intensively than do those of the MFGM. While a common origin of the skim milk and fat globule phospholipids may exist, the differences in the palmitate labeling patterns (Table I, Fig. 2) indicate that phospholipids from the two sites equilibrate quite differently with the tracer, and that at some point they have diverged biosynthetically.

Membranes of Golgi vesicles, which are vehicles in the secretion of the milk proteins and which interact with the plasma membrane at secretion are a logical source of the skim milk lipoprotein. The occurrence of Golgi membranes in skim milk is also suggested by the presence of UDP galactosyl transferase (18,19). This enzyme, the A protein of lactose synthetase (19), is localized in the Golgi apparatus of lactating mammary tissue (20) and represents a marker for the Golgi apparatus in hepatocytes (21,22). The shedding of plasma membrane or of some MFGM components into the skim milk are not precluded by the present findings.

The probability that the nonfat phase of milk contains small amounts of cell membrane in a physiologically dispersed state offers a unique research opportunity. Preliminary experiments (D.L. Puppione and S. Patton, unpublished) reveal the presence of skim milk lipoprotein in the ultracentrifugal density class 1.063-1.21 g/ml.

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Free Radicals, Malonaldehyde and Protein Damage in Lipid-Protein Systems

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ABSTRACT

The free radical concentration in lipid-protein mixtures with a low moisture content was monitored both during and after the time that the lipid actively absorbed oxygen. The data show that, in dry systems, the decay in radical content is followed by a rise in malonaldehyde protein fluorescence. Analysis of amino acid content at two distinctly different periods in the reaction substantiate the hypothesis that radicals and not aldehydes are a major cause of protein damage.

INTRODUCTION

In studies of lipid-protein nucleotide interaction, a number of investigations have shown that polyunsaturated fatty acids undergoing oxidation can induce extensive changes in enzymes, proteins and nucleotides (1-8). In some of these studies, radicals derived from oxidizing lipid have been implicated as the chief cause of damage. It is therefore of some importance to consider lipid oxidation, radicals and their involvement in biological systems in detail. Although there is ample evidence that lipid oxidation is a chain reaction involving free radical intermediates, limitations in instrumental sensitivity make it most unlikely that radicals derived from lipid can be readily characterized in native biological material or in any system high in moisture content (1).

Free radicals are a chief cause of the observed damage, i.e., destruction of amino acids of proteins or enzymes, as well as polymerization or depolymerization of nucleotides. However, aldehydes or other nonradical lipid oxidation products may also be capable of causing damage to such biopolymers. The purpose of this investigation was to ascertain which mechanism, radical or nonradical, dominates in systems containing protein in close association with oxidizing lipid.

EXPERIMENTAL PROCEDURES

Electron Paramagnetic Resonance of Dry Biological Systems

To detect and study radicals and radical lifetime, solid state systems were used because, in these, line widths are not too broad and the lipid-derived radical steady state concentration is high enough to make detection by electron paramagnetic resonance (EPR) an easy matter. In contrast to the study of radicals in solution, however, EPR of many solids is complicated by the fact that the observed EPR spectrum will consist of a superposition of absorbancies arising from all orientations of immobilized radicals. The worst situation would be one in which anisotropies in both the g-value and hyperfine splitting would spread the EPR absorption line over hundreds of gauss. If this were to happen, the line would probably be so badly broadened that observation would be almost impossible. Fortunately, with most biological samples, organs, tissue, etc., such extreme broadening is not encountered. In fact, the absorptions are, for the most part, fairly narrow, on the order of 3-11 gauss.

Lipids, Proteins and Test Mixtures

The fatty acid mixture used in all studies consisted of approximately 75% (by weight) of C22:6 and approximately 25% C20:5. Rockfish myofibrilliar protein, freeze dried and extracted with isopropyl alcohol and hexane, and then dried in vacuum, was supplied by the NMFS Technological Laboratory in Seattle. Bovine serum albumin (BSA; lyophillized) was obtained from the Sigma Chemical Corporation.

Lipid-protein test mixtures were prepared merely by stirring together lipid and dry protein (1:2 w/w) for 1-2 min. Except for the buffering capacity of protein itself, no attempt was made to control the pH of the lipid-protein mixtures. Samples were allowed to oxidize under oxygen at 37 C and sampling was begun 2 hr after mixing. All samples for EPR study were analyzed at room temperature as described previously (9). Amino acid analyses were performed on hydrolysates (conventional 16 hr hydrolysis in HCl in sealed tubes) by the National Center for Fish Protein Concentrate, College Park, Maryland, using an amino acid analyzer.

Chloroform-methanol (2:1 v/v) extracts of solid reaction products were scanned for fluorescence using an Aminco-Bowman spectrophotofluorimeter equipped with a solid state blank substract photometer.

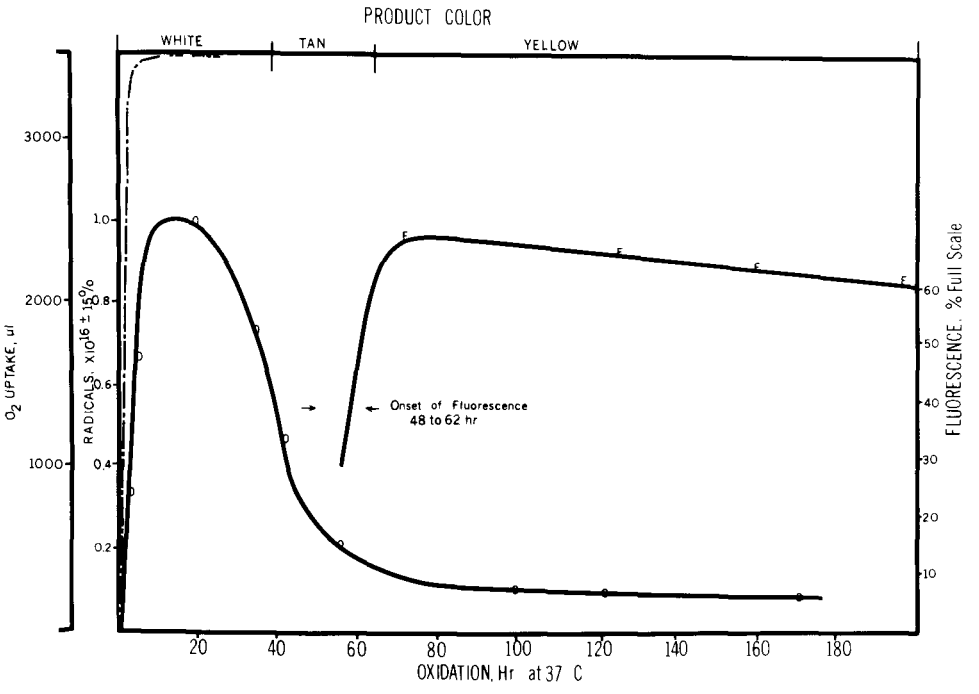

FIG. 1. Radical content, Schiff base fluorescence and product color vs. time of lipid oxidation in bovine serum albumin - C22:6. F, fluorescence; O, radical content; —— - —— -, oxygen uptake. Measurable oxidation, as monitored by oxygen uptake, ceased rather abruptly at about 8 hr under an excess of oxygen. Ratio of lipid-protein, 1:2 w/w.

Butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) (Eastman Chemicals), in separate tests, were included in some lipid-protein mixtures in order to obtain information concerning the effects of these two popular antioxidants. They were added to protein along with lipid at a level amounting to twice the amount of lipid (on a mole basis).

RESULTS AND DISCUSSION

In a previous study (9) it was shown that, in addition to a dominant central EPR signal in the g=2 region (a central resonance not directly associated with lipid oxidation), there appeared, in those protein samples containing oxidizing or oxidized lipid, one and sometimes two additional peaks downfield from the central resonance. The nature of these lipid signals has been discussed. Since radicals could be immobilized, yet slowly decay (unpublished work by the author has recently shown that the rate of decay is dependent on the amount of lipid present and on the type of protein), the hypothesis that radicals would give way to fluorescence as malonaldehyde reaction products accumulate was tested. Indeed, the data of Figure 1 for lipid-BSA, show a rapid accumulation of radicals during the time that lipid actively absorbs oxygen. For the first day or two, chloroform-methanol extracts (no added antioxidant), were devoid of spectral characteristics indicative of malonaldehyde-amino acid interaction (formation of N,N'-disubstituted 1-amino-3-iminopropene fluorescence: $\lambda max_{excitation}$=390 mμ, $\lambda_{fluorescence}$ = 470-480 mμ). Figure 2 shows the relation between length of oxidation and specific fluorescence. Also, as shown in Figure 1, characteristic iminopropene fluorescence is detected in the very same time period that there is a marked decrease in observable radical content. When iminopropene fluorescence did appear, fluorescence intensity did not change significantly but remained more or less constant throughout the remainder of the examination (7-10 days). Myofibrilliar protein in place of BSA gave similar results. The greatest difference was in the somewhat shortened time required to produce measurable fluorescence.

In an attempt to facilitate analyses, fluorescence of solid reaction mixtures themselves was investigated. However, it has been our experience, and that of others, that solid samples, however appealing they may be for direct fluorescence measurement, are not satis-

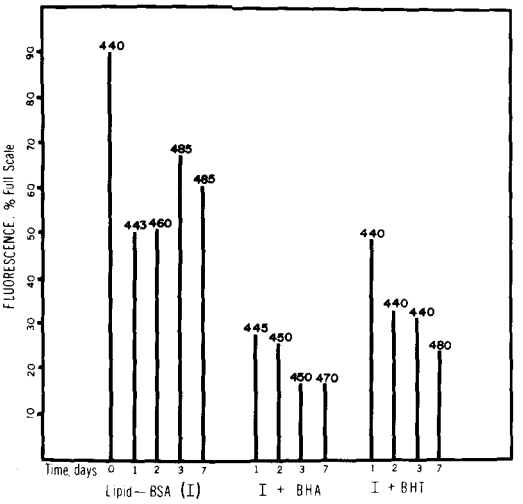

FIG. 2. Effect of added antioxidant on retardation of Schiff base formation. Numbers at tops of columns are fluorescent wave length maxima for chloroform-methanol extracts excited at 390 mμ. BSA, bovine serum albumin; BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene.

factory. Thus all fluorescence data was obtained using chloroform-methanol extracts. (Tappel, for instance, points out that this solvent appears to be the appropriate mixture for selective removal of malonaldehyde-amino reaction products.)

When BHT or BHA were present (Fig. 2), the onset of fluorescence was delayed and it was not until the seventh day that iminopropenes could be detected. Although fluorescence did occur eventually, the fluorescence intensity was less than that for samples devoid of antioxidants. Although not shown in the figures, BHT or BHA, though effective in reducing onset of fluorescence, did not completely suppress the EPR lipid signal. BHT-treated material gave a weak recorded resonance after a few hours of oxidation and the signal was still detectable at 12 hr. Only a trace of EPR signal was ever detected in BHA-treated samples, an observation that correlates with a somewhat less intense fluorescence noted for BHA data in Figure 2. The eventual formation of fluorescence in antioxidant-treated material can only mean that a low but undetectable steady state concentration of radicals is presented.

Amino acid analyses are significant for they correlate well with time course observations of trapped radicals and fluorescence. With BSA for instance, a 14 hr period represents the approximate midpoint in time at which radical content is maximum. On the other hand, a 72 hr period represents a time at which radicals have decayed appreciably but fluorescence is maximum. Although all amino acids suffered damage, its extent was not as great as that observed in previous studies of protein-lipid-aqueous buffer systems. Losses ranged from 20% to 40% for the 14 hr period. Methionine, cystine, tyrosine, alanine and lysine suffered 30%, 40%, 34%, 27% and 22% losses, respectively. However, in the next 58 hr, the losses for these same amino acids were only 15%, 18%, 15%, 15% and 10%, respectively.

Compared to samples consisting of lipid-protein-aqueous emulsions, an overall lowering in the losses of amino acids is probably due in part to lipid polymerization with subsequent loss, as reactant, of the thin lipid layers deposited on proteins. However, this hypothesis cannot be entirely correct because fluorescence occurs at a time considerably displaced from the cessation of uptake of oxygen.

The data indicate that major losses in amino acids occur rather early in the oxidation. Some of the loss at a later period may not be due to aldehydes but may still be the result of a small but detectable radical content. Although Chio and Tappel (6,7) have only recently given a thorough account of malonaldehyde-protein/enzyme interaction, their data do not provide information on the significance of aldehydes in the chain of events which transpire as lipid oxidation progresses first to the free radical state and then to malonaldehyde and other nonradical reactants. The results of the present study, therefore, further strengthen the hypothesis first introduced by Roubal and Tappal (1,3) that radical attack and not aldehyde attack on protein is predominately responsible for damage to proteins.

Although the present study indicates that food grade antioxidants appear to act as inhibitors of lipid signals, that is, trapped radicals, additional research will be required to clarify the role and mode of action of antioxidants in foods exhibiting EPR signals.

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SHORT COMMUNICATION

Cholesterol Interference in Analysis of Fatty Acid Methyl Esters

ABSTRACT

In the course of isolating and analyzing the fatty acid methyl esters (FAME) from steelhead trout (*Salmo gairdneri*) eggs and sac fry, a component was found on gas liquid chromatography to have a retention time longer than any characteristic polyunsaturated fatty acid previously encountered in trout. Subsequent purification and analysis demonstrated this component to be cholesterol. Thus, cholesterol and related compounds can interfere in the analysis of FAME when the methyl esters are prepared by transesterification.

A common method in use for preparation of fatty acid methyl esters (FAME) from lipid samples is that of transesterification using anhydrous HCl in methanol-ether (1:1), followed by extraction with a nonpolar solvent such as hexane. This method is rapid and complete, and is used extensively (1-4). While using this procedure in our laboratory in the preparation of FAME from steelhead eggs and sac fry, a component was encountered which possessed a long retention time (8.6 with respect to methyl stearate) and had not been encountered previously in this lab in sufficient quantity for identification (the present samples contained 2-5% of total FAME). Neither had a similar component been reported in the fatty acids of *Salmo gairdneri* (5,6).

Although several investigators have reported 24 carbon fatty acids in the fat of marine and fresh water fish (7-9), the most commonly reported acid being 24:1, it became apparent that the component isolated here was not an ordinary fatty acid. Upon hydrogenation the retention time becaue shorter, but did not correspond to either 24:0 or 23:0. Separation from the other components of the FAME sample was easily accomplished by thin layer chromatography (TLC) on Silica Gel G, using dichloromethane as solvent. Rate of flow, R_f for the unknown was .3, similar to that of hydroxy-fatty acids, whereas the remainder of the FAME moved with the solvent front. In this manner, sufficient quantity was obtained for further analysis.

The IR spectrum (Beckman Microspec, double beam, in CCl_4) gave major absorptions at 2.8, 3.4, 6.8, 7.2, and 9.5 μ. Conspicuously absent was an ester absorption at 5.8 μ. Mass

TABLE I

Ion Peak Intensity, as Per Cent of 386 (Molecule-Ion)

m/e	FAME Component[a]	Cholesterol Standard[a]	Friedland et al. Cholesterol
387	36	40	---
386	100	100	100
384	13	08	08
372	22	19	---
371	61	72	50
369	27	28	---
368	68	79	106
354	27	34	---
353	70	84	82
302	20	27	---
301	70	98	55
276	32	40	---
275	109	132	108
274	32	32	---
273	52	58	33
255	61	77	60
247	38	40	42

[a]FAME component and cholesterol standard, partial mass spectra.

spectral analysis (Varian Mat CH7 mass spectrometer, 70 eV, 100μa, 100C, direct injection) indicated a molecule-ion peak at m/e 386, with a distribution of m/e signals very similar to that reported for cholesterol (10) and corresponding very closely to that of a sample of cholesterol run on the same instrument (Table I).

The chromatographic behavior of the unknown and of cholesterol were identical on TLC and gas liquid chromatography (GLC). TLC on Silica Gel G, developed with 40% ether in hexane, produced identical R_f values for both compounds. Upon spraying with Liebermann-Burchard reagent and heating at 100 C for 10 min both standard and unknown exhibited a characteristic deep rose color. GLC on a 6' x 1/8" OD aluminum column of 15% diethylene glycol succinate on AW Chromosorb P, 80-100 mesh at 190 C, 19 psi and 30 ml/min, utilizing solid sample injection (11), resulted in identical retention times for the unknown and the cholesterol standard. Under these operating conditions 5 μg were readily detected.

It is assumed that the inclusion of steroids as contaminants in a fatty acid methyl ester preparation does not usually interfere with their analysis by GLC (12). This assumption is based on their long retention times and thermolabile nature. A large dead space between the injection site and column inlet promotes decomposition, particularly when the sample is injected in solution (13). The fact that we have obtained interference from cholesterol could be due in part to the use of a solid sample injector which provides "on column" injection and minimizes the problem due to dead space. In addition, those conditions which give shorter retention times and better peak definition of the long chain polyunsaturated fatty acids would also make it more likely that cholesterol would give a well defined peak and be mistaken for a fatty acid. This interference can be eliminated by applying the FAME sample to a silicic acid column and eluting with 3% ether in hexane, or by a saponification step.

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Composition of Wax Esters, Triglycerides and Diacyl Glyceryl Ethers in the Jaw and Blubber Fats of the Amazon River Dolphin (*Inia geoffrensis*)

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ABSTRACT

The lower jaw fat of the Amazon River dolphin *Inia geoffrensis* contains 52.8% wax ester, 44.7% triglyceride and 2.5% diacyl glyceryl ether, while its dorsal blubber fat is > 98% triglyceride. Examination of the intact lipids, the derived fatty acids and the derived fatty alcohols by gas chromatography reveals that the blubber triglycerides show characteristics of freshwater fish fats, but the jaw fat lipids have several distinctive features. Jaw fat wax esters, triglycerides and diacyl glyceryl ethers are all rich in C_{10}, C_{12} and C_{14} fatty acids and contain no polyunsaturated acids. The fatty alcohols in the wax esters are over 90% saturated. The major carbon numbers in the jaw fat triglycerides (C_{38}-C_{46}) are considerably lower than those of the blubber triglycerides (C_{48}-C_{54}). The possible adaptation of the jaw lipids for use in the underwater echolocation process of this dolphin is discussed.

INTRODUCTION

The order Cetacea is divided into two suborders on the basis of dentition. The suborder Mysticeti (baleen whales) possesses jaws with fibrous baleen plates which are used to filter small animals from the water for food. The suborder Odontoceti (toothed whales, dolphins and porpoises) have normal teeth and capture their food with a biting action. Except for the commercially important sperm whale, the Odontoceti were virtually ignored in scientific studies for many years. Recently, however, many of the smaller species have become aquarium attractions and are used as exceptionally intelligent experimental animals for the study of respiration and other physiological processes, including audiocommunication and echolocation (1-8).

The most primitive group of animals within the Odontoceti are the family Platanistidae comprising four genera of dolphins living mostly in freshwater rather than marine habitats. *I. geoffrensis* Blainville (9), the sole *Inia* species and a typical member of the Platanistidae, is found in the upper Amazon and Orinoco Rivers far from the open sea. An echolocation system appropriate to the muddy river waters in which it lives might assist selective hunting and feeding (3). Caldwell, et al. (7) have described sound production and associated behavior in captive *Inia* which suggest that the animal is capable of echolocation. As part of a study of the properties and involvement of lipids in Cetacean sound organs, and in marine mammals generally, we have investigated the lipid compositions of the dorsal blubber and of the lower jaw fat (a possible ultrasonic sound receptor) in *I. geoffrensis*. No previous studies of *Inia* lipids have been reported in the literature.

MATERIALS AND METHODS

Materials

The materials studied were removed from a 1.403 m long adult female *I. geoffrensis* by W.P. Perrin, U.S. Bureau of Commercial Fisheries, La Jolla, California. The animal was captured in 1969 in the San Fernando de Apure River in Venezuela and died during air transfer to Sea World in San Diego, California.

The sample of blubber, from the area immediately in front of the dorsal fin, had skin attached and was about 20 mm thick. It was sectioned into inner and outer layers of equal thickness. The fat from the lower jaw had no skin or apparent blubber attached. The fats were recovered from these samples by extracting in a Waring blendor with hexane, drying over sodium sulfate and stripping off all solvent under high vacuum ($<100\ \mu$). The recovered oil samples had the following Wijs iodine values: inner blubber, 87.5; outer blubber, 93.3; jaw fat, 33.5.

The blubber oil in bulk was solid at 24 C, softened at 25 C, liquified from 26 to 27 C and cleared finally at 30 C. On cooling it became hazy at 29 C but was still liquid down to 25 C. The jaw oil had more sharply defined thermal

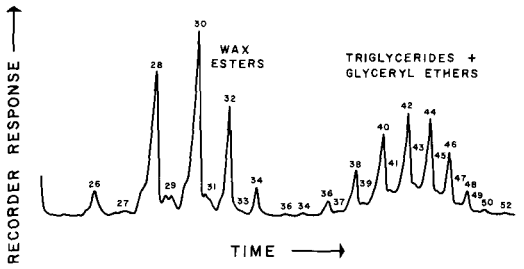

FIG. 1. Gas chromatogram of intact lipids from *Inia geoffrensis* lower jaw fat. Peaks are labeled according to carbon number. Operating conditions: 560 x 2.5 mm i.d. column packed with 3% JXR on 100/120 mesh Gas-Chrom Q, 100 ml/min He, column temperature programmed from 160 C-360 C at 4 C/min, injection port 350 C, F & M 400 gas chromatograph.

characteristics. It softened at 28 C, was viscid at 29 C and cleared completely at 31 C. On cooling it was clear down to 26 C, viscid at about 25 C and became a solid mass at 24 C. Both liquid oils were water white.

Analysis of Intact Lipids

Each sample was hydrogenated by the method of Farquhar et al. (10) using freshly distilled dioxane in lieu of ethanol. The hydrogenated lipids were then subjected to preparative thin layer chromatography (TLC) on 1.0 mm thick layers of silicic acid (Adsorbosil-1, Applied Science Laboratories) developed with petroleum ether-diethyl ether-acetic acid (87:12:1). The blubber lipid was almost entirely triglyceride, but the jaw lipid was fractionated into three spots having the R_f values of wax ester, diacyl glyceryl ether and triglyceride. Each band was individually scraped from the plate, placed in a small chromatography column, and the lipid recovered by elution with diethyl ether. Two additional preparative TLC separations of the jaw fat were also carried out, one in which the wax ester and diacyl glyceryl ether bands were combined and recovered as a single sample, and another in which the wax ester and triglyceride bands were recovered together.

The intact lipids from each TLC fraction were analyzed by gas liquid chromatography (GLC) in the manner described by Litchfield et al. (11) for triglycerides. Samples were run on an F&M 400 gas chromatograph fitted with a flame ionization detector (FID) and a 560x2.5 mm i.d. stainless steel column packed with 3% JXR silicone on 100/120 mesh Gas-Chrom Q. The column was linearly programmed from 160 to 360 C at 4 C/min with an injection port temperature of 340-350 C and 100 ml/min He carrier gas. Comparison of each sample with

TABLE I

Lipid Class Composition of *Inia geoffrensis* Lower Jaw and Dorsal Blubber Fats

Lipid class	Lower jaw, wt %	Dorsal blubber, wt %
Wax ester	52.8	---
Triglyceride	44.7	>98
Diacyl glyceryl ether	2.5	---

authentic wax ester, triglyceride and diacyl glyceryl ether standards confirmed the identities assigned from TLC results. Chromatograms were quantitated in the usual manner (11) based on calibration mixtures of 99+% pure wax esters (Analabs), triglycerides (Applied Science Laboratories) or diacyl glyceryl ethers (Analabs). Peak areas were measured with a planimeter. Comparison of the average carbon number of the intact molecules with the average chain lengths of the derived fatty acids and fatty alcohols (11) gave values differing by less than 2%, confirming the general accuracy of the carbon number distributions reported. Since the wax esters were completely eluted from the GLC column before the overlapping triglyceride and diacyl glyceryl ether peaks (Fig. 1), it was possible to use GLC of the intact lipids to determine the amount of each lipid class present. Gas chromatograms of the wax ester plus triglyceride and of the wax ester plus diacyl glyceryl ether TLC fractions were analyzed separately. Since the wax esters were common to both chromatograms, the relative amounts of wax ester, triglyceride and diacyl glyceryl ether could be readily calculated from the combined results.

Analysis of Fatty Acids and Fatty Alcohols

A portion of each blubber oil was saponified, and the nonsaponifiables were removed by a standard AOCS procedure (12). The inside blubber oil contained 0.6 wt % and the outside blubber oil 0.4% nonsaponifiable material. The recovered fatty acids were converted to methyl esters with boron trifluoride-methanol reagent.

The wax esters isolated from the jaw lipids by TLC were saponified, the fatty alcohols were extracted with diethyl ether and the alcohols were converted to acetates by reaction with acetic anhydride. The jaw triglycerides were converted to methyl esters with boron trifluoride-methanol reagent. The diacyl glyceryl ethers from the jaw fat were saponified and the recovered nonsaponifiable portion was converted to trimethylsilyl (TMS) ethers for GLC. The fatty acids from the diacyl glyceryl ethers

were also recovered and converted into methyl esters. Parts of all methyl ester and alcohol acetate samples were hydrogenated for additional GLC study.

GLC analyses of all methyl ester, alcohol acetate and TMS ether samples were carried out on a Perkin-Elmer 226 gas chromatograph fitted with a FID and 46 m x 0.25 mm i.d. open-tubular (capillary) columns. The injection port was operated at 250 C. Primary analyses were accomplished on columns coated with butanediolsuccinate polyester operated at 170 C and 50 psig He. These results were supplemented in certain cases by appropriate analyses on Apiezon L coated open-tubular columns at 190 C and 80 psig. The glyceryl ether TMS derivatives were also examined on a 1.8 m x 3.2 mm o.d. stainless steel column packed with 3% SE-30 on Chromosorb W and operated at 210 C and 30 psig in a Varian-Aerograph Hi-Fy gas chromatograph. Quantitation of methyl esters and alcohol acetates was based on corrected FID peak areas from Disc integrator results (13) and verified by examination of hydrogenated samples (14). Following quantitation, results were converted from weight per cent to mole per cent fatty acid. For the glyceryl ether TMS derivatives, area per cent was equated with weight per cent. Presentation of data to two decimal points is solely to show certain small components and the quantitative errors are probably of the order of ±5% for major components, ±10% for minor components and proportionally higher for trace components.

RESULTS AND DISCUSSION

Lower Jaw Fat

The lower jaw fat of *I. geoffrensis* contains approximately equal portions of wax ester and triglyceride, together with a small amount of diacyl glyceryl ether (Table I). The possible relationship of this lipid class composition to the physiology of echolocation is discussed below.

Wax Esters. The wax ester alcohol moiety is based predominantly (64.2 mole %) on cetyl alcohol (hexadecanol, 16:0a) [The usual shorthand notation of (chain length): (number of double bonds) ω (position relative to terminal methyl group) is used for fatty acids, and also for fatty alcohols with the addition of the suffix "a".] As shown in Table II, the rest of the alcohol components are qualitatively more or less those anticipated with the exception of 9.4 mole % iso 16:0a. A very recent review of wax esters in marine organisms includes GLC data which does not suggest the likelihood of as high a proportion of iso 16:0a in other marine invertebrates or vertebrates, including some marine mammals (15). The low level of monoethylenic fatty alcohols is perhaps unexpected. In the blubber of the Atlantic bottlenose whale (*Hyperoodon ampullatus* Forster) and the blubber of the sperm whale (*Physeter catodon* Linnaeus), 18:1a may be about half of the wax alcohols and 6-10% 16:1a has also been recorded (16,17). The C_{18} monoethylenic fatty alcohols (18:1aω9 and 18:1aω7) are assumed to be *cis* and their double bond positions were identified by comparison with fatty alcohols prepared from whole herring oil, studied previously (18). The ratio is that commonly found in marine oils and lipids (19). The corresponding C_{16} alcohols are low in the tentatively identified ω7 isomer relative to the adjacent isomers.

The fatty acid moiety of the wax esters (Table II) differs from most of the other wax esters of marine origin mentioned above in the high proportions of acids with chain length $<C_{14}$. However, 5-8 wt % 10:0 and 20-21 wt % 12:0 have been found in sperm whale head oil waxes (20-22) and are mentioned for the possibly associated sperm spinal cord waxes (23). In any event, they are less evident in sperm body oil (1% for 12:0) than in head oil. The ratio of iso 16:0 to 16:0 is nearly the same in the fatty acids of the three lipids of the *Inia* jaw fat, suggesting a common fatty acid pool sharply differentiated from the blubber fats where iso 16:0 is only 1/100th of 16:0. The ratios of iso 16:0 and 16:0 in the wax ester alcohols and acids are so close that they also suggest a common endogenous origin or free interconversion as discussed elsewhere (15,24,25). The similar ratios of 18:0 to 18:1 and 18:0a to 18:1a also support this view (Fig 2 and 3).

During this investigation, particular attention was paid to the exotic minor fatty acids currently under study by two of the authors. *Trans*-6-hexadecenoic acid (26) was absent. Among the isoprenoid fatty acids (27), phytanic acid was 0.05% or less, and pristanic and 4,8,12-trimethyltridecanoic acids were barely detectable. The wax ester fatty alcohols from the jaw fat contained absolutely no dihydrophytol. This virtual absence of the isoprenoid fatty acids in all the *Inia* lipids examined (Table II) is in strong contrast to the 0.4-1.6% of some marine mammal fats (27), or to the 0.2-0.7% of temperate zone freshwater fish fats (28). No explanation of this is apparent to us.

The combination of mainly C_{10}-C_{16} fatty acids with C_{14}-C_{18} fatty alcohols results in

TABLE II

Compositions (Mole %) of Lipid Fractions From *Inia geoffrensis* Lower Jaw and Dorsal Blubber

Shorthand structure notation[d]	Triglycerides	Lower jaw				Dorsal blubber	
		Fatty acids[b]		Fatty alcohols[c] from wax esters	Glyceryl ethers[d] from diacyl glyceryl ethers	Fatty acids[b]	
		Wax esters	Diacyl glyceryl ethers			Outer blubber	Inner blubber
Iso 10:0	0.29	0.64	0.48	---	---	---	---
10:0	3.00	6.56	3.23	---	---	0.28	0.14
Iso 11:0	0.36	0.59	0.44	---	---	---	---
Anteiso 11:0	0.31	0.30	0.24	---	---	---	---
11:0	0.44	0.71	0.68	---	---	---	---
Iso 12:0	2.76	3.64	3.44	---	---	0.17	0.03
12:0	18.51	25.21	16.65	---	---	1.60	0.98
Iso 13:0	0.42	0.39	0.36	---	---	0.19	0.06
Anteiso 13:0	0.32	0.39	0.27	---	---	0.17	0.03
13:0	0.97	1.30	0.95	---	---	0.36	0.20
Iso 14:0	4.28	3.36	4.80	---	---	0.33	0.14
14:0	27.90	17.08	22.34	6.68	8.55	6.75	5.64
Iso 15:0	0.78	1.02	1.02	0.80	---	0.72	0.45
Anteiso 15:0	0.58	0.55	0.87	---	---	0.33	0.31
15:0	0.85	0.73	1.19	2.86	3.91	0.96	0.78
Iso 16:0	1.79	0.37	1.07	9.43	---	0.19	0.17
16:0	17.01	4.19	15.01	64.18	44.77	17.92	24.66
Iso 17:0	0.15	0.09	0.10	0.77	---	0.55	0.36
Anteiso 17:0	0.12	0.05	0.05	0.44	---	0.10	0.08
17:0	0.44	0.11	0.19	1.06	4.09	0.88	0.78
Iso 18:0	0.10	0.07	0.08	---	---	0.05	0.06
18:0	0.55	0.64	1.19	4.18	6.64	2.45	3.82
19:0	Trace	Trace	Trace	---	---	0.14	0.08
20:0	---	---	---	---	---	0.05	0.06
Total saturated	81.93	68.19	74.65	90.40	67.96	34.19	38.83
12:1	2.63	7.85	3.74	---	---	0.47	0.14
12:1	0.96	1.24	0.53	---	---	0.22	0.08
14:1	1.65	7.20	2.91	---	9.83	0.85	0.36
14:1	1.54	2.40	1.75	---	---	0.94	0.53
14:1	1.50	2.02	1.75	---	---	1.85	1.20
14:1	0.99	4.80	2.91	---	---	---	---
15:1	0.10	0.66	0.15	---	9.28	0.06	0.06

Unknown	0.24	1.01	0.41	---	---	0.17	0.11
16:1ω9	1.60	0.73	1.94	0.49	4.19	1.60	1.06
ω7	5.74	3.25	6.45	0.67	---	23.13	17.38
ω5	0.07	Trace	Trace	0.13	---	0.30	0.39
17:1ω8	0.05	Trace	0.15	---	2.09	0.69	0.70
18:1ω11 + 9	0.77	0.75	2.30	5.99	6.64	19.37	23.30
ω7	0.22	0.09	0.29	2.36	---	3.03	2.93
ω5	Trace	Trace	0.02	---	---	0.19	0.14
19:1ω10	---	---	---	---	---	0.03	0.06
ω 9	---	---	---	---	---	0.03	0.03
ω 8	---	---	---	---	---	---	0.08
20:1ω11	---	---	---	---	---	0.08	0.22
ω 9	---	---	---	---	---	0.44	1.30
ω 7	---	---	---	---	---	---	0.37
22:1ω13 + 11	---	---	---	---	---	---	---
Total monoene	18.06	32.00	25.30	9.64	32.03	53.45	50.44
16:2ω6	---	---	---	---	---	0.44	0.31
ω4	---	---	---	---	---	0.03	0.03
ω?	---	---	---	---	---	0.22	0.06
16:3ω4	---	---	---	---	---	0.08	0.06
3ω3	---	---	---	---	---	0.25	0.22
16:4ω3	---	---	---	---	---	0.08	Trace
4ω1	---	---	---	---	---	---	---
18:2ω6	---	---	---	---	---	5.45	6.61
3ω6	---	---	---	---	---	0.08	0.11
3ω3	---	---	---	---	---	2.59	1.48
4ω3	---	---	---	---	---	0.03	Trace
20:2ω6	---	---	---	---	---	0.11	0.22
3ω6	---	---	---	---	---	0.88	0.31
3ω3	---	---	---	---	---	0.28	0.22
4ω6	---	---	---	---	---	1.10	0.64
4ω3	---	---	---	---	---	0.17	0.08
20:5ω3	---	---	---	---	---	0.14	0.08
21:4ω2	---	---	---	---	---	---	---
22:4ω6	---	---	---	---	---	0.06	0.08
22:5ω6	---	---	---	---	---	0.11	0.06
22:5ω3	---	---	---	---	---	0.14	0.08
22:6ω3	---	---	---	---	---	0.11	0.08
Total polyunsaturated	---	---	---	---	---	12.35	10.73

[a]Chain length: unsaturation and position relative to terminal methyl group.

[b]Determined as methyl esters.

[c]Determined as acetates.

[d]Determined as trimethylsilyl ethers.

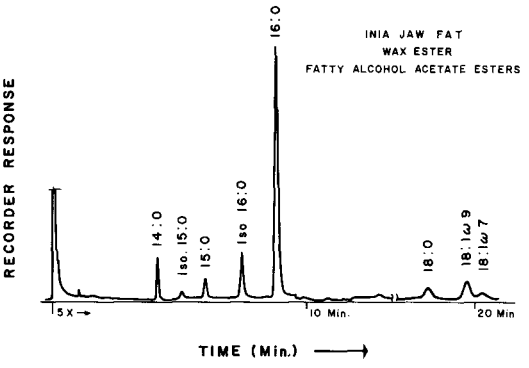

FIG. 2. Gas chromatogram of acetate esters of fatty alcohols from *Inia* lower jaw wax esters. Operating conditions: 46 m x 0.25 mm i.d. open-tubular column coated with butanediolsuccinate polyester, 170 C, 50 psig He, Perkin-Elmer Model 226 gas chromatograph. Attenuation 5x throughout.

most of the wax esters having carbon numbers between 26 and 32 (Fig. 1, Table III). No C_{19}-C_{23} species (i.e., isovaleroyl wax esters) were found, although they were specifically looked for. The average wax ester molecular weight is lower than the C_{30}-C_{38} range found in most marine wax esters (15,16,29,30). If one calculates a random distribution of the fatty alcohols and fatty acids found in *Inia* lower jaw wax esters (Table III), the distribution of carbon numbers is very similar to that found experimentally. This indicates a definite lack of specificity in the *Inia* enzyme system performing this esterification, a phenomenon that has been noted in some (15,29,30) but not all (30) other wax esters in marine organisms. The front shoulders on the wax ester GLC peaks (Fig. 1) are no doubt caused by the branched chain acids and alcohols found in these compounds (Table II), since branched chain esters are known to elute slightly before corresponding straight chain esters on silicone columns (31,32).

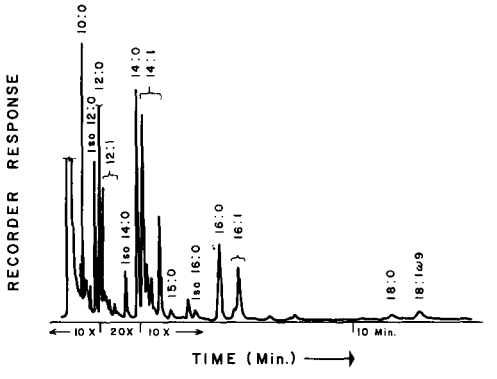

FIG. 3. Gas chromatogram of methyl esters of fatty acids from *Inia* lower jaw wax esters. Operating conditions same as Fig. 2. Attenuations (x) marked at bottom.

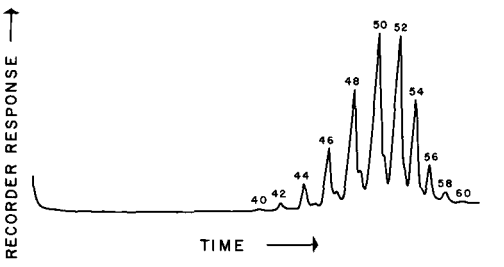

FIG. 4. Gas chromatogram of intact triglycerides from *Inia geoffrensis* outer dorsal blubber. Peaks are labeled according to carbon number. Operating conditions are the same as in Figure 1.

Triglycerides. The fatty acids found in the lower jaw triglycerides qualitatively resemble those of the lower jaw wax esters, i.e., mostly C_{10}-C_{16} chain lengths, and a complete absence of polyunsaturated species. Quantitatively, however, the triglyceride acids have a slightly higher average molecular weight. Myristic is the major acid in the triglycerides, while lauric acid predominates in the wax esters.

Gas chromatograms of the intact lower jaw triglycerides (Fig. 1, Table III) show that most of the molecules fall in the C_{38} through C_{46} range. This is a much lower range of carbon numbers than the C_{48}-C_{60} triglycerides mixtures found in most aquatic organisms (11,31,32), including the triglycerides from *Inia* blubber. Asymmetric peaks are again encountered because of the presence of substantial amounts of even chain length iso acids, which slightly decrease triglyceride retention times (31,32).

Diacyl Glyceryl Ethers. The saturated glyceryl ethers of *Inia* lower jaw fat (Table II) resemble the wax ester fatty alcohols to a surprising degree, but with the omission of the iso forms. This suggests an ostensible origin of the glyceryl ether chain for this particular system (33), but this is an area not without controversy or several alternate possibilities (34,35). The monoethylenic and saturated TMS derivatives did not separate on polyester columns (36), and the evidence for the monoethylenic structures rests largely on analyses of two different nonpolar lipid phases, where complete GLC coincidence of suspected and reference selachyl (18:1) TMS derivatives was obtained. Impurities in this reference material correspond to 14:1 and 16:1 structures and also coincided with *Inia* lipid peaks. When retention data was plotted, the odd-numbered monoethylenic components lay on the same line as the even-numbered, and all were con-

TABLE III

Carbon Number Distribution (Mole %) of Wax Esters, Triglycerides and Diacyl Glyceryl Ethers From *Inia geoffrensis* Lower Jaw and Dorsal Blubber Fats

Carbon number	Lower jaw				Dorsal blubber	
	Wax esters				Inner	Outer
	Found	Calc.[a]	Triglycerides	Diacyl glyceryl ethers	Triglycerides	
24	Trace	0.5	---	---	---	---
25	Trace	0.4	---	---	---	---
26	5.7	8.0	---	---	---	---
27	2.1	2.9	---	---	---	---
28	30.5	31.9	---	---	---	---
29	4.6	4.2	---	---	---	---
30	35.8	33.1	---	---	---	---
31	2.6	3.7	---	---	---	---
32	14.7	11.9	---	---	---	---
33	0.5	0.8	---	---	---	---
34	3.2	2.3	0.6	---	---	---
35	Trace	0.1	---	---	---	---
36	0.3	0.2	3.5	---	---	---
37	---	---	0.5	---	---	---
38	---	---	10.6	1.3	---	---
39	---	---	0.9	0.3	---	---
40	---	---	20.3	9.7	0.2	0.2
41	---	---	1.1	3.5	---	---
42	---	---	24.5	22.0	0.6	0.8
43	---	---	1.1	4.0	Trace	0.1
44	---	---	20.4	25.8	2.0	2.6
45	---	---	0.8	3.3	0.5	0.6
46	---	---	10.4	17.7	6.0	7.2
47	---	---	0.5	1.3	0.9	1.2
48	---	---	3.7	8.6	15.9	17.0
49	---	---	0.3	---	1.2	1.7
50	---	---	0.6	2.5	26.5	26.4
51	---	---	---	---	1.4	2.0
52	---	---	0.2	---	23.9	23.6
53	---	---	---	---	1.0	0.8
54	---	---	---	---	12.4	10.9
55	---	---	---	---	0.7	0.5
56	---	---	---	---	4.5	3.4
57	---	---	---	---	0.2	0.1
58	---	---	---	---	1.8	0.8
60	---	---	---	---	0.3	0.1

[a]Calculated assuming a random esterification of fatty acids with fatty alcohols.

verted to the expected saturated form on hydrogenation. Lack of sample precluded further studies. It is evident that the proposed minor monoethylenic and saturated glyceryl ethers occur in about the same order of magnitude (2-10% mole %) except for chimyl (16:0). Some minor (ca. 1%) glyceryl ethers such as 17:1 have been reported in an elasmobranch marine oil (37), but we are aware of only one case where an unusual odd-numbered glyceryl ether is reported in large proportions (up to 17.4 wt %) in a marine (elasmobranch) lipid (38). However there is no reason to expect that the *Inia* jaw fat glyceryl ether will conform to either poikilotherm or terrestrial mammal patterns.

The fatty acids from these diacyl ethers are very similar in gross composition and in detail to those of the triglycerides except for some increase in the C_{18} and monoethylenic acids.

GLC of the intact diacyl glyceryl ethers revealed a C_{40}-C_{48} carbon number distribution very similar to that of the lower jaw triglycerides (Table III). This range of molecular weights is considerably lower than the mainly C_{48}-C_{58} diacyl glyceryl ether mixture reported for tumor lipids (39).

Dorsal Blubber Fat

The difference in iodine value (IV) between the inner (IV=87.5) and outer (IV=93.3) blubber layers is quite small compared to the reverse gradient observed for North Atlantic finwhales (40), where the inner layer had a

value of 114.8 and the outer 89.9. Correspondingly, the differences in fatty acid composition are slight (Table II).

Inia blubber fat bears a close resemblance to the blubber fat of the Ganges River dolphin *Platanista gangetica* Lebeck, the only other member of the Platanistidae whose lipids have been investigated (42). Levels of saturated C_{12}, C_{14}, C_{16} and C_{18} and unsaturated C_{14}, C_{16}, C_{18} and C_{20} acids are very similar in both species, but *Platanista* shows 2-3 mole % more unsaturated C_{22}. However, since the *Platanista* analysis was performed by the Pb-Li salt precipitation-distillation procedure, a detailed comparison of individual acids is not possible.

The details in respect to the saturated C_{12} and C_{13} acids, the common n-15:0 and n-17:0 acids and the C_{14}-C_{18} iso and anteiso acids in *Inia* blubber fat are quite ordinary for marine oils, such as cod liver oil (14), or for freshwater fish oils (28). The monoethylenic isomer distributions possibly show some emphasis on 16:1ω9 and 18:1ω9 relative to ω7 isomers. For marine oils there is also the somewhat unusual plurality of obvious 12:1 and 14:1 isomers. Active desaturation of a number of saturated acids in the 9,10 position and subsequent free chain length interconversions might account for these. The triglycerides of sperm blubber oil contain appreciable C_{20} and C_{22} acids in the form of 20:1 at 15 wt % and 22:1 at 12% (20), which are notably absent in the *Inia* blubber. The very low proportions of polyunsaturated C_{20} and C_{22} fatty acids has a parallel in sperm blubber oil (41).

The differences from marine blubber fats (17,40,cf.14) are however, quite interesting in that 18:2ω6 (but not 18:3ω6) and the successor acids 20:3ω6 and 20:4ω6 are quite prominent; whereas, although 18:3ω3 is obviously of some importance in the C_{18} polyunsaturates, successor acids such as 18:4ω3 and those of higher chain lengths are negligible. This emphasis on C_{16} (and C_{18}) chain length, and on ω6 fatty acids relative to ω3, accords with the freshwater environment as discussed elsewhere for freshwater fish (42,43). The virtual absence of C_{20} and C_{22} polyunsaturated fatty acids is unusual in aquatic animal triglycerides. Curiously, among fish which contain wax esters, the livers of coelacanth (*Latimeria chalumnae*) and castor oil fish (*Ruvettus pretiosus*) also have virtually no polyunsaturated fatty acids in the triglycerides (29,44), but lantern fish triglycerides (from whole fish or muscle) contain the moderate proportions expected in fish oils (45).

The GLC pattern for intact triglycerides from the blubber fat (Fig. 4, Table III) shows mainly C_{46} through C_{54} components, reflecting the predominance of C_{16} and C_{18} acids and the very low levels of C_{20} and C_{22} acids. This C_{46}-C_{54} range of blubber triglycerides is considerably higher than the mainly C_{38}-C_{46} triglycerides found in *Inia* lower jaw fat (Fig. 1).

Although the blubber of *I. geoffrensis* has some affiliations with freshwater fish lipid fatty acid composition, from most points of view *Inia* blubber is a unique triglyceride, even for marine mammals. This is probably because of the mode of life, based on the constant availability of food, and because the absence of prolonged migrations, characteristic of the lives of marine cetaceans (1), does not require a large fat reserve. The environmental temperature is probably relatively warm and constant compared to corresponding conditions for marine animals. An active cetacean produces considerable heat, and we have found that as little as a few millimeters of blubber is observed in some smaller Odontoceti (Ackman et al., unpublished). This and other factors, such as vascular heat exchange systems (1,46), require less desk work and more field investigation and experiment on the species in question (46).

Implications for Odontocete Echolocation

The present results on *Inia* lower jaw fat can be correlated with the lipids found in the melon and jaw fats of other toothed whales. In all cases investigated so far, the Odontocete fatty tissues implicated in the echolocation function contain mostly triglycerides [*Delphinapterus leucas* (Litchfield et al., unpublished), *Phocaenoides dalli* (47,48), *Phocoena phocoena* (49)] or a mixture of mainly wax esters and triglycerides [*I. geoffrensis*, *Globicephala melaena* (50), *Tursiops truncatus* (51), *Physeter catodon* (20-22), *Orcinus orca* (52)]. In each case in which a comparison was made, the fatty acids found in the melon and jaw fats have a significantly lower average chain length than those found in the blubber of the same animal. In *Inia* and *Physeter*, this means higher levels of C_{10}-C_{14} fatty acids, while in the other genera there is a large amount of isovaleric acid in the wax esters and triglycerides. In either case, it seems likely that the lower molecular weight lipid molecules and the presence of wax esters confer advantageous physical properties to the fatty tissues which may be involved in ultrasonic sound transmission and reception. Whether this is due to unique acoustical properties or to the ability of the lipid to remain liquid at environmental temperatures remains to be seen. Studies now in progress on the melon, jaw and blubber fats of other Odon-

tocete species may clarify the physiological role of the unusual head lipids found in these animals.

The possible function of 2.5% diacyl glyceryl ethers in *Inia* jaw fat remains obscure as it does in most tissues where this lipid is found (34). It seems unlikely, however, that they are present in sufficient quantity to have any major effect on the acoustical properties of the fatty tissue.

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Isotope Effects in the Desaturation of Stearic to Oleic Acid

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ABSTRACT

Comparisons have been made of the rates of desaturation of stearic to oleic acid using a series of differently labeled forms of the substrate and two different methods of assay. The reaction has been measured either by analyzing the amount of labeled oleic acid produced, or by analyzing the amount of tritium released into water from [9,10-$^3H_2$]-labeled substrates. The rate of desaturation of [1-^{14}C] stearic acid has been compared with that of [*threo*-9,10-$^3H_2$] and [*erythro*-9,10-$^3H_2$] stearic acids in which about 99% of the label was at the 9 and 10 positions and in which most of the labeled molecular species contained two tritium atoms. A further series of [*erythro*-9,10-$^3H_2$] stearates was used in which there was a greater proportion of tritium at positions other than 9 and 10 or a greater proportion of species containing only one tritium atom, or both. In the tritium release assay the enzyme discriminated against the tritium substrates, the discrimination being greater with *erythro*-ditritio-compounds than with monotritio or *threo*-ditritio-compounds. The isotope effect was also observed when [^{3}H] stearoyl-CoA thiol esters were the substrates, and when the source of the enzyme was the green alga *Chlorella vulgaris*. Despite the isotope effect, the release of tritiated water can be used as an assay of desaturase activity. If the absolute value of enzymic activity is required, however, the location and stereochemistry of the tritium atoms should be known or the method standardized against [1-^{14}C] stearic acid.

INTRODUCTION

Until recently, estimation of the extent of enzymic desaturation of long chain fatty acids involved a time-consuming sequence of procedures, e.g., saponification, extraction, methylation, chromatographic separation and radioassay (1,2). A quick, reliable and reproducible method was required particularly in studies involving the solubilization and purification of desaturases (3-5). In 1969, Talamo and Bloch (6) published a procedure which apparently fitted all these criteria. This method depends on the fact that tritium at the 9 and 10 positions of stearic acid is released as tritiated water during enzymic desaturation. The protein and associated lipid are precipitated with trichloroacetic acid and measurement of tritium in the clear aqueous solution after filtration provides a measure of the amount of unsaturated acid formed.

Two published studies on the mechanism of desaturation had already pointed out that an isotope effect occurred, resulting in a slower rate of removal of tritium (7) or deuterium (8) than hydrogen from the 9 and 10 positions. Before attempting to use the tritium release assay as a routine procedure, therefore, it was considered necessary to ascertain the magnitude of the isotope effect and to what extent it might limit the use of the assay.

Preliminary experiments, in which mixtures of [1-^{14}C] stearic acid and [9,10-$^3H_2$(n)] stearic acid were used as substrates, indicated that the amount of desaturation as measured by release of tritium into water was much less than that measured by conversion of [1-^{14}C] stearic acid into oleic acid. This paper describes a series of experiments carried out to investigate this isotope effect and to determine the validity of the assay method under a wide range of conditions.

MATERIALS AND METHODS

Assay Procedure

Measurement of desaturase activity by conversion of labeled stearic acid into oleic acid, separation of saturated and monounsaturated methyl esters by argentation thin layer chromatography (TLC) and their radioassay by scintillation spectrometry have already been described (1).

Measurement of desaturase activity by release of tritium from [9,10-$^3H_2$] stearic acid into water was carried out by the method of Talamo and Bloch (6), except that the material precipitated by TCA from the incubation mixture was centrifuged instead of being filtered through a Millipore filter. Preliminary experiments indicated that the enzymic desaturation reaction was linear for at least 30 min and this time was used in all standard incubations. All

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assays were done at least in duplicate and usually in triplicate. Substrates were added as albumin complexes. The acid was first dispersed by sonication in 0.2 M phosphate buffer, pH 7.4, and bovine serum albumin added to a concentration of 48 mg/ml. The specific activity of [1-^{14}C] stearate was 48.6 μCi/μmole and of all tritiated substrates, 500 μCi/μmole. In a total volume of 0.5 ml, the incubation mixture contained the following: fatty acid in varying concentrations; phosphate buffer, 0.2 M; coenzyme A, 134 μM; ATP, 16 mM; NADH, 934 μM; NADPH, 400 μM; $MgCl_2$, 3.3 mM; protein, about 15 mg.

After addition of 1.2 ml trichloroacetic acid and centrifugation of the precipitate, 1 ml of the supernatant was removed for radioassay in 15 ml toluene containing 13% Bio-Solv (Beckmann Instruments Inc., Fullerton, Calif.) and 2% Butyl-PBD (Koch-Light Limited, Colnbrook, England). For lipid samples, 2% Butyl-PBD in toluene was used. Radioactivity was measured with a Philips Automatic Liquid Scintillation Analyzer.

Preparation of Cell Fractions

White shaver hens, 12-18 months old, were fed a high protein, high carbohydrate diet which had the following fatty acid composition: 14:0, 1%; 16:0, 15%; 16:1, 1%; 18:0, 2%; 18:1, 23%; 18:2, 55%; 18:3, 1%; others, 2%. All experiments with hen liver employed a 15,000 x *g* x 30 min supernatant fraction (2). Protein was measured by a biuret method (9). Cell free homogenates of *Chlorella vulgaris* were prepared as previously described (10).

Substrates

All radiochemicals were purchased from the Radiochemical Centre, Amersham, England. Table I illustrates the variety of tritiated substrates used. All except A (Amersham TRA 13, Batch 9, made by tritiation with a nonspecific catalyst) were made by essentially the same two-step process: (a) reduction of octadec-9-ynoic acid (stearolic) with tritium gas in the presence of a specific partially poisoned catalyst to give [9,10-$^3H_2$] oleic acid; (b) further reduction to give [9,10-$^3H_2$] stearic acid. Substrate B is the commercial product, Amersham TRA 13 (Batch 10). The first reduction was done with carrier free tritium gas, thus yielding a product in which virtually all the labeled molecular species are *erythro*-ditritio. Substrate C was made for us commercially, the initial reduction having been done with a 50% mixture of hydrogen and tritium so that, on a statistical basis, it should consist of monotritio species and *erythro*-ditritio species in the ratio, 2:1. Substrate D ([*erythro*-9,10-$^3H_2$] stearic acid) was made in our laboratory by reduction of Amersham [9,10-$^3H_2$] oleic acid (TRA 140, Batch 5) with hydrazine hydrate in acetonitrile (11), and substrate E ([*threo*-9,10-$^3H_2$] stearic acid) was made by first isomerizing [9,10-$^3H_2$] oleic acid with oxides of nitrogen (12) followed by hydrazine reduction of the [9,10$^3H_2$] elaidic acid isolated by argentation TLC. Substrates C, D and E were purified by argentation TLC prior to use. The percentage of tritium at the 9 and 10 positions was measured by G.K. Koch, Unilever Research Laboratorium, Vlaardingen, The Netherlands (13).

Calculation of Results

The calculation of the extent of desaturation as measured by release of tritium into water is complicated by two factors: (a) In each substrate there is a certain proportion of tritium which is not in the 9 and 10 positions (Table I). This figure has been calculated from oxidation experiments (above). (b) A second correction has to be made and arises out of the stereochemistry of desaturation. Only the pair of hydrogen (or tritium) atoms with the D-absolute configuration are removed during the enzymic reaction (7,8) and therefore, as the substrates comprise exactly equal amounts of D and L forms, only half of the 9,10-tritium can possibly be removed during desaturation.

The following formulae were used to calculate the results:

Number of nmoles of substrate = S

$$= \frac{\text{dpm in substrate}}{2.2 \times 10^6 \times \text{specific activity}} \times \frac{\%\ \text{tritium in 9,10-positions}}{100} \quad [1]$$

Number of nmoles of product = P

$$= \frac{\text{dpm in product - dpm in zero-time control}}{2.2 \times 10^6 \times \text{specific activity}} \quad [2]$$

Per cent tritium from 9,10-position released into water

$$= \frac{P}{S} \times 100 \quad [3]$$

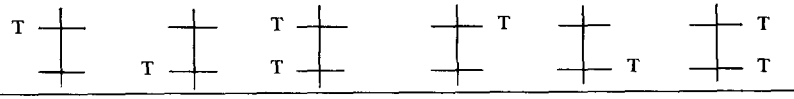
Per cent desaturation by tritium release assay

$$= \frac{P}{S} \times 100 \times 2 \quad [4]$$

In these formulae, the specific activity equals 0.5 μC/nmole. Zero-time control values were: Substrate A, 0.60%; B, 0.50%; C, 0.10%;

TABLE I

Location of Tritium in the Stearic Acid Substrates

Stearic acid	Stereochemistry at $C_{9,10}$	Per cent tritium at $C_{9,10}$
A. [*Erythro*–9,10-$^{3}_{-}$H$_2$(n)][a]		45
B. [*Erythro*–9,10-$^{3}_{-}$H$_2$(n)]		60
D. [*Erythro*–9,10-$^{3}_{-}$H$_2$]		99
E. [*Threo*–9,10-$^{3}_{-}$H$_2$]		99
C. Mono [9-$^{3}_{-}$H] or [10-$^{3}_{-}$H]		78

[a]The radiochemical abbreviation (n) indicates the nominal position of the isotope when there is any uncertainty as to whether the labeling is confined to the nominal position.

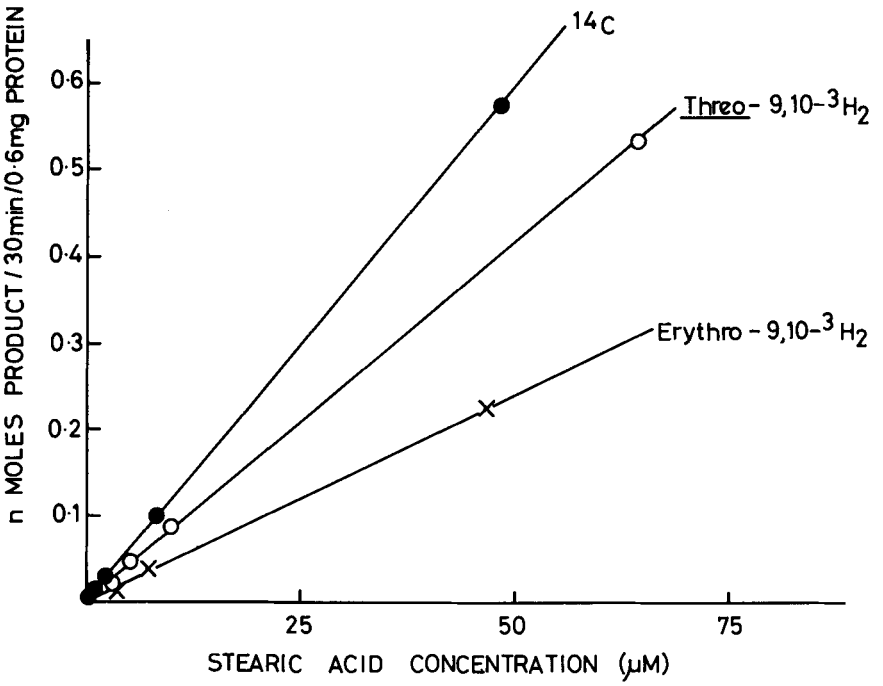


FIG. 1. The kinetics of oleate formation from [1-^{14}C] stearic acid and of tritiated water formation from [*threo*-9,10$^{3}H_2$] and [*erythro*-9,10$^{3}H_2$] stearic acids. The values for tritiated water are corrected for the percentage of tritium not at the 9 and 10 positions (Table I) and for the stereospecificity of the desaturase reaction. The method of calculation of these results is illustrated in Methods (Formula 4).

D,E, 0.15% of the radioactivity of the substrate. The factor 2 in Formula [4] allows for the fact that only hydrogen (or tritium) atoms having the D-absolute configuration will be removed during desaturation. *Threo* substrates (Table I) consist entirely of species containing one D-tritium and one L-tritium atom and, therefore, the maximum number of tritium atoms which can possibly be released into water is one half of the total number of tritium atoms in the molecules. *Erythro* substrates (Table I) consist of an equal mixture of species having either two D-tritium or two L-tritium atoms. Tritiated water can only be produced from species containing a D-tritium atom, i.e., half the total number of tritiated molecules, whereas the other 50% of molecules release hydrogen into water, which cannot be detected. Therefore the absolute desaturation of each substrate will be twice that measured by maximum theoretical tritium release.

RESULTS

Kinetic Studies

One of the methods for studying the kinetics of an enzyme reaction involves increasing the substrate concentration to saturate the enzyme and thus estimate the maximum rate of the enzymic reaction (V_{max}). In the present case, there are two possible substrates for the enzyme, the one with hydrogen atoms at the 9 and 10 positions and the other with tritium atoms at these positions. To study the kinetics of desaturation of the latter, it is necessary to use substrates of high specific activity and very high levels of radioactivity. To minimize the hazards and cost involved, the normal desaturase preparation was diluted 10-fold. Even so, it was not possible to fully saturate the enzyme.

Figure 1 shows a comparison of the rate of formation of [1-^{14}C] oleic acid from [1-^{14}C] stearic acid, with the rates of formation of tritiated water from [*erythro*-9,10-$^{3}H_2$] and [*threo*-9,10-$^{3}H_2$] stearic acids (substrates D and E respectively) as the substrate concentrations are increased. The statistical method of Wilkinson (14) was used to calculate V_{max} and apparent K_m values for each substrate (Table II). The expression "apparent K_m" is used because more than one enzyme is involved in the desaturation of fatty acids, and because the exact concentration of substrate is unknown,

TABLE II

Kinetic Data[a] for the Desaturation of Three Different Forms of Stearic Acid

Stearic acid	V_{max} nmoles/ 30 min/0.6 mg protein	Apparent K_m x 10^4M
[1-^{14}C]	7.5	5.7
Threo-di-T(E)	4.6	4.9
Erythro-di-T(D)	2.3	4.3

[a]Corrected for (a) the presence of tritium which is in positions other than 9,10 (Table I) and (b) for the stereochemistry of desaturation (see text.) The method of Wilkinson (14) was used to calculate V_{max} and K_m.

TABLE III

Comparison of the Amount of Tritium Found in Water and Oleic Acid After Enzymic Desaturation of Different Substrates

Stearic acid	Per cent tritium in: H_2O[a]	Oleic acid
E. *Threo*-di-T	13.2	17.9
C. Mono-9T or 1-T	14.3	28.3
D. *Erythro*-di-T	6.3	24.6
B. *Erythro*-di-T	8.6	25.1
A. *Erythro*-di-T	8.4	26.3

[a]Corrected for the presence of tritium which is in positions other than 9 and 10 (Table I). The method of calculation of these results is shown in Methods (Formula 3). [1-^{14}C] stearic acid desaturation was 40%.

due to the possible formation of micelles and the uncertainty regarding the amount of endogenous substrate. The magnitudes of the apparent K_m values are in the same order as the maximum velocities, namely ^{14}C>*threo*-9,10-$^3H_2$>DD-*erythro*-9,10-$^3H_2$, indicating that two tritium atoms at the 9 and 10 positions are removed more slowly than one tritium atom and one hydrogen atom, which in turn are removed more slowly than two hydrogen atoms. However, the apparent K_m values indicate that the strengths of binding of the substrates to the enzyme are in the following order. DD-*erythro*-9,10-$^3H_2$>*threo*-9,10-$^3H_2$>^{14}C

The amount of tritium in the product, oleic acid, depends on the nature of the substrate and the extent of desaturation. For example, the amount of tritium released into water from the [*threo*-9,10-$^3H_2$] substrate (E) should be exactly equal to the amount of tritium in the oleic acid formed, if 100% of the labeled species were ditritio, the tritium atoms were truly in the *threo* configuration, and 100% of the tritium atoms were at the 9 and 10 positions. This must be so, whether or not an isotope effect exists. Table III confirms that the amounts of radioactivity in water and oleic acid are similar. In contrast, the oleic acid formed from the [*erythro*-9,10-$^3H_2$] substrate (D) contained a much larger proportion of tritium than is released into water. This material consists essentially of an equal mixture of two substrates. The first, in which the D-9 and D-10 positions are occupied by hydrogen atoms, will be desaturated at the normal rate assuming that there is no significant secondary kinetic isotope effect. No tritiated water will be produced and the oleic acid formed will contain all the tritium of the substrate. The second, in which the D-9 and D-10 positions are both occupied by tritium atoms, will be desaturated more slowly due to the primary kinetic isotope effect. Thus, oleic acid will contain more tritium than the water (Table III). When the tritium at positions other than 9 or 10 is taken into account, the amount of tritiated water from substrates A and B, in which the 9,10-tritium atoms are also largely in the *erythro* configuration, is similar to that from substrate D. The calculation of the percentage desaturation as measured by [^{3}H] oleic acid formation is complicated by the relatively high proportion of tritium not at the 9 and 10 positions in these substrates. Substrate C is a mixture of *erythro*-ditritio and monotritio forms in the ratio, 1:2. As the monotritio form is itself a mixture, (since the tritium can be located at any of four positions, Table I), it is equivalent to the *threo* configuration. Thus, substrate C shows both effects in that the amount of tritium in water is almost the same as that derived from the *threo* substrate, while the tritium content of the oleic acid is more similar to that of an *erythro* substrate.

To summarize these results: two hydrogen atoms are released more rapidly than one hydrogen atom and one tritium atom, which in turn are released more rapidly than two tritium atoms from the D-9 and D-10 positions of stearic acid during desaturation. The relative rates of release are approximately: HH/HT/TT, 3:2:1.

The isotope effect also operated when the tritiated substrates were in the form of the stearoyl-CoA thiol esters, and also when the source of the desaturase was *Chlorella vulgaris*.

Validity of the Assay

The validity of this procedure as an assay for desaturase depends on the strict proportionality between the amount of tritium released into

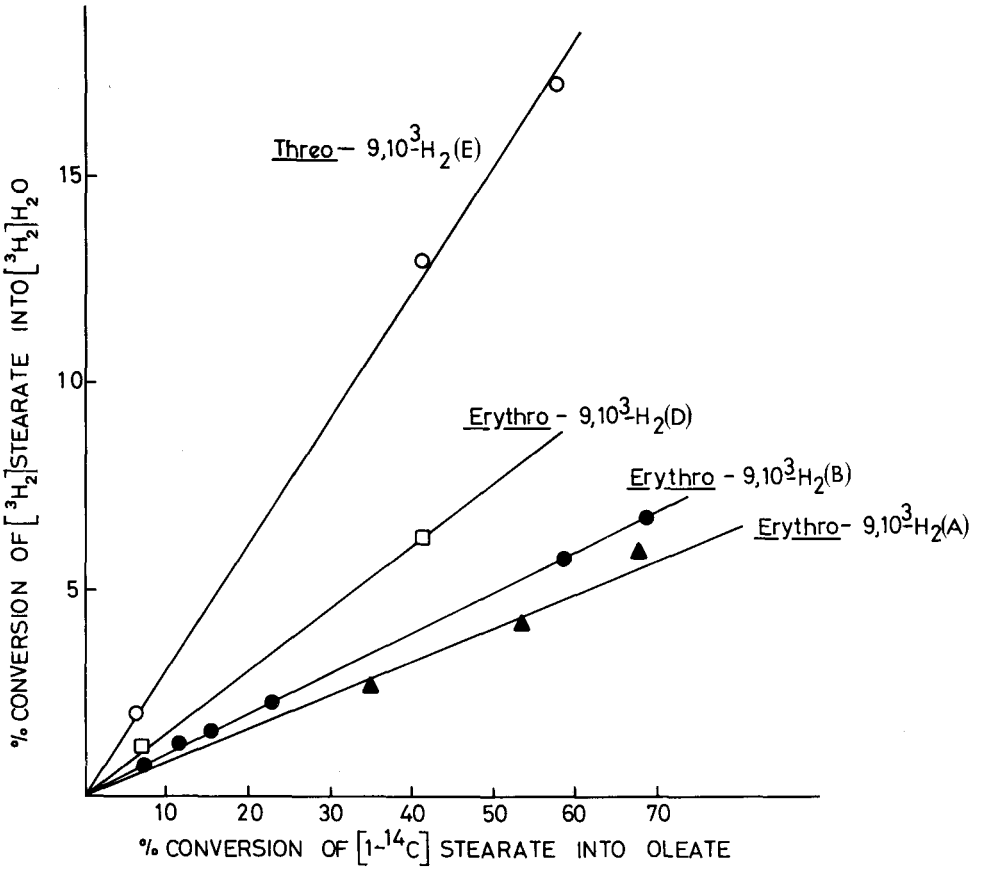


FIG. 2. The validity of the assay procedure over a wide range of conditions. The values for per cent conversion of [$^3H_2$] stearate into tritiated water are not corrected in any way in order to show than any substrate gives a linear relationship between tritium release and desaturation.

water and the amount of oleic acid formed as measured by the conversion of [1-^{14}C] stearic acid into oleic acid. The data from a large number of experiments covering a wide range of conditions and three different tritiated substrates are plotted in Figure 2, as percentage conversion of stearate into oleate against percentage of tritium from the substrate released into water, and demonstrate, in fact, that such a proportionality does exist.

DISCUSSION

The results presented in this paper can be discussed from two different viewpoints. First, there is the question of the validity of the assay. The procedure can only be a true measure of desaturation if the formation of tritiated water is strictly proportional to oleate formation over a wide range of different conditions, such as different enzyme preparations with widely differing activities, at different extents of reaction, and with different substrates. Figure 2 shows that this proportionality does exist over a wide range of different conditions. If the absolute value of desaturase activity is required, each individual tritiated substrate must be calibrated against [^{14}C] stearic acid. The isotope effect reduces the sensitivity of the assay but this can be minimized by using [*threo*-9,10-$^3H_2$] stearic acid (E) or the monotritio substrate (C), having the substrate of high specific activity, and taking particular care to obtain low zero time control values.

The other question concerns what these data can tell us about the mechanism of desaturation. Schroepfer and Bloch (7) made the four stereospecifically labeled 9- and 10-monotritiostearic acids and showed that the 9- and 10-D tritium atoms were removed during desaturation, whereas the 9- and 10-L tritium atoms were not. They observed an isotope effect at the 9 position only and suggested that desatu-

ration proceeded by a stepwise removal of hydrogen, first at the 9 position as the rate-limiting step, then by removal at the 10 position. In contrast, Morris et al. (8) found an isotope effect against deuterium at both positions. They argued for a mechanism in which both hydrogens were removed simultaneously. With the present data, we still cannot distinguish between a stepwise or a concerted mechanism. However, the isotope effect involved in the removal of the two tritium atoms at the 9 and 10 positions was twice that for the removal of one tritium at either the 9 or 10 positions (Table II), indicating an isotope effect at both positions, thus confirming the work of Morris et al. (8).

The apparent K_m values listed in Table II indicate that binding of the DD-*erythro*-ditritio substrate to the enzyme is stronger than that of the *threo*-compound, which in turn is stronger than that of [1-^{14}C] stearate. The firmer binding of tritiated substrates could result in an inhibition by [9,10-$^{3}H_2$] stearic acid on the desaturation of [1-^{14}C] stearic acid. In addition, the higher the proportion of tritiated species present, the stronger the binding and the slower the release of tritium into water. Caution must be exercised in interpreting results of any double-label experiments especially when either of the labeled atoms is directly involved in the reaction (15).

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Sterol Metabolism: XII. 26-Hydroxycholesterol in Commercial Cholesterol[1]

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ABSTRACT

Commercial cholesterol of bovine brain and spinal cord origin has been carefully examined for its 24- and 26-hydroxycholesterol content. By alternate adsorption chromatography and chromatography on Sephadex LH-20 26-hydroxycholesterol was isolated from this course in 15 μg/g amounts, whereas 24-hydroxycholesterol was not observed.

INTRODUCTION

Our studies of trace level polar sterols in human tissue (1-3) suggest that specific trace sterols may accumulate in select tissues but not in others. Thus, human aortal tissue contains 26-hydroxycholesterol as a major trace sterol (1,2,4,5), but human brain does not (3). Human brain contains the 24-hydroxycholesterol cerebrosterol (3,6,7), but human aortal tissue is devoid of this sterol (1). The apparent differential accumulation of specific hydroxycholesterol derivatives in separate human tissues prompted us to reexamine the possible occurrence of the aortal sterol 26-hydroxycholesterol in brain tissues where larger amounts of sterol were available for study. In the present report we demonstrate the presence of 26-hydroxycholesterol in bovine spinal cord and brain cholesterol to the extent of 15 μg/g.

EXPERIMENTAL PROCEDURES

Melting points were taken on a calibrated Kofler block under microscopic magnification. IR absorption spectra were recorded over the range 400-4000 cm^{-1} with a Perkin-Elmer Model 337 spectrophotometer equipped with a beam condenser. Samples were incorporated into 1.5 mm diameter potassium bromide disks prepared with a micro die.

All solvents employed in chromatography were redistilled shortly before use. Adsorption chromatography was conducted on columns using silica gel (Baker Analyzed, reagent grade). Preparative chromatography on Sephadex LH-10 (Pharmacia Fine Chemicals Inc., Uppsala) was conducted with methylene chloride in the manner described previously in detail (8).

Thin Layer Chromatography

Thin layer chromatography (TLC) was conducted on 20 x 20 cm chromatoplates of Silica Gel HF_{254} 0.25 mm thick (E. Merck GmbH., Darmstadt), irrigated chiefly with benzene-ethyl acetate (3:2) using techniques previously described (9). TLC mobility data are given in terms of cholesterol as a unit mobility. Preparative TLC was conducted on 20 x 20 and 20 x 40 cm chromatoplates 1 and 2 mm thick of Silica Gel PF_{254} irrigated with benzene-ethyl acetate (3:2). Sample application was by means of the Rodder Streaker (Rodder Instrument Co., Los Altos, Calif.). Steroids were routinely detected by their UV light absorption properties on the phosphor-coated chromatoplates and with 50% aqueous sulfuric acid as a spray. On heating, the sprayed chromatoplates displayed characteristic colors for the several sterols.

Gas Chromatography

Gas chromatographic analyses were conducted on 3% SE-30 and 3% QF-1 columns using Hewlett-Packard F and M Model 400 and 402 gas chromatograms, as described previously in detail (10). Gas chromatographic mobility data are expressed as relative retention times (t_R) with cholesterol as unit time.

Cholesterol Samples

A 1 kg batch of commercial cholesterol (Wilson Laboratories, Division of Wilson Pharmaceutical Corp., Chicago) of established age of five years after manufacture was recrystallized repeatedly from methanol to give crops of crystalline cholesterol free from autoxidation artifacts and companion sterols by TLC and gas chromatography (9,10). Each crop was recrystallized from methanol and the methanol mother liquor added to the initial mother liquor. Concentration of the mother liquor under vacuum afforded additional crops of crystalline cholesterol free of artifacts. This process was continued until pure cholesterol could no longer be recovered. About 75% of the initial sample of cholesterol was recovered by this means.

[1]Paper XI of this series: J.E. van Lier and L.L. Smith, Steroids 15:485-503 (1970).

Fractionation Procedures

The methanol mother liquors were concentrated under vacuum to yield a mobile oil containing a complex mixture of sterols. The mixed sterols were chromatographed on a 2.5 x 50 cm column of silica gel prepared in benzene. Elution with 4 liters of benzene gave fractions containing cholesterol and cholesta-3,5-dien-7-one, together with other apolar autoxidation products. Elution with 2 liters of benzene-ethyl acetate (4:1) gave three fractions which were characterized: Fraction 1, mainly cholesterol with autoxidation products more mobile than 25-hydroxycholesterol; Fraction 2, a sterol diol fraction containing 25-hydroxycholesterol and 26-hydroxycholesterol; Fraction 3, sterols more polar than 25-hydroxycholesterol, including 5α-cholestane-3β,5,6β-triol and material which did not migrate in the usual TLC systems used for analysis.

Fraction 1 contained some 25-hydroxycholesterol and was accordingly recrystallized from methanol to remove cholesterol, and the mother liquor was rechromatographed on silica gel with the same solvent systems initially used. The sterol diol Fraction 2 therefrom was pooled with the initial sterol diol Fraction 2 and the pooled sterol diols were chromatographed on a 2.5 x 50 cm column of Sephadex LH-20 developed with methylene chloride (20 ml fractions collected automatically). 25-Hydroxycholesterol was eluted in tubes 25-50 (peak tube 35), and 26-hydroxycholesterol was eluted in tubes 45-60 (peak tube 55). Tubes 25-45 were evaporated under vacuum and crystallized from ethyl acetate, yielding 1.6 g of 25-hydroxycholesterol, mp 172-176 C, not depressed on admixture with authentic 25-hydroxycholesterol, variously reported with mp 181.0-182.0 C (1), 181.5-182.5 C (11), 177-179 C (12), 179-180 C (13), 175-177 C (14), 178-180 C (15), 179-183 C (16), 177-178.5 C (17). In all cases the 25-hydroxycholesterol recovered was rigorously identified by IR absorption spectra, TLC and gas chromatography, and was shown to be free from other sterol diols by these means.

Fractions 45-60 were combined and chromatographed on three 2 mm thick chromatoplates of Silica Gel PF_{254} using benzene-ethyl acetate (3:2). The zone containing both 25- and 26-hydroxycholesterol, unresolved in this system, was scrapped from the plates and packed into a small glass column which was eluted with methanol-chloroform (1:2). The mixed sterol diols were rechromatographed on a 1 x 60 cm column of Sephadex LH-20 using methylene chloride. 25-Hydroxycholesterol, eluted ahead of the 26-hydroxycholesterol band, did not significantly overlap the sought 26-hydroxycholesterol fraction under these conditions. Recovery of the 26-hydroxycholesterol and rechromatography on Sephadex LH-20, followed by TLC and recrystallization from hexane-diethyl ether afforded 15 mg of pure 26-hydroxycholesterol, free from 25-hydroxycholesterol or other demonstrable component, mp 169-171 C, not depressed on admixture with authentic (25R)-26-hydroxycholesterol. (25R)-26-Hydroxycholesterol derived from kryptogenin is reported with mp 177-178 C (18,19); (25RS)-26-hydroxycholesterol of synthesis origin is reported with mp 168-173 C (20). Identity of the sample with the reference samples was further established by comparison of IR spectra ($\tilde{\nu}_{max}^{KBr}$ 3300, 1620, 1050 cm^{-1}), by TLC (R_C 0.49, magenta color with 50% sulfuric acid), and gas chromatography (t_R 3.24 on 3% QF-1, 2.26 on 3% SE-30). For comparison, chromatographic properties of 25-hydroxycholesterol are: R_C 0.57, purple-blue color; t_R 2.38 on 3% QF-1, 1.64 on 3% SE-30.

Confirmation of these results was obtained with a second complex sterol diol fraction derived by us in other work from an extensively autoxidized batch of cholesterol (21). The sterol diol fraction was resolved by alternate chromatography on silica gel and Sephadex LH-20 by the same means as described above, yielding 25- and 26-hydroxycholesterol, identified by comparison with the reference samples. The level of 26-hydroxycholesterol was the same for both cholesterol samples; however, the 25-hydroxycholesterol level was much higher in the extensively autoxidized batch (15 g/kg).

DISCUSSION

In these studies we have established that 26-hydroxycholesterol is present in commercial cholesterol samples of bovine brain and spinal cord origin at the 15 μg/g level. In distinction we did not uncover evidence at any point for the presence of the 24-hydroxycholesterol cerebrosterol associated with human and equine brain. However, the autoxidation artifact 25-hydroxycholesterol, known to be formed readily from cholesterol in the solid state (9,14,22), was present at 100- to 1000-fold levels (1.6-15 mg/g) over that of 26-hydroxycholesterol. In our prior studies on human aortal tissue the 26-hydroxycholesterol levels exceeded those of 25-hydroxycholesterol (1), and we conclude that the elevated 25-hydroxycholesterol levels in the commercial cholesterol

samples represents autoxidation by air over variable periods of time.

In other studies we have isolated cholesterol 25-hydroperoxide from cholesterol subjected to autoxidation in air (21), and we have more recently demonstrated the thermal decomposition of cholesterol 25-hydroperoxide to give, among other steroids, 25-hydroxycholesterol (23). The status of 25-hydroxycholesterol as an artifact of air oxidation of cholesterol is now fully established.

The cases of the 24-hydroxycholesterol of human and equine brain and the 26-hydroxycholesterol of the human aorta suggest that neither be of artifact nature, but that both be derived from a metabolic origin. The brain sterol appears to be a stereochemically pure 24-hydroxycholesterol (7) to which a $24\beta_F$ (24S)-configuration has been assigned (24). Accordingly, an enzymic origin is indicated.

Configuration assignments have not been made for the 26-hydroxycholesterol samples derived from human aortal tissue or from bovine brain and spinal cord. The bovine 26-hydroxycholesterol sample melted sharply at 169-171 C in distinction to the human aortal sample which melted at 172.5-174.0 C (1). Both samples melt below the (25R)-26-hydroxycholesterol (mp 177-178 C) derived from kryptogenin (18) and more nearly correspond in melting behavior to the (25RS)-26-hydroxycholesterol (mp 168-173 C) prepared by synthesis (20). However, (25S)-26-hydroxycholesterol has not been described, and melting behavior cannot be confidently for assignment of configuration in its absence. Although configurational purity cannot be utilized in this instance in support of a probable enzyme origin for tissue 26-hydroxycholesterol, we regard the sterol as being enzymatically derived and have presented arguments on the matter previously (1,3).

26-Hydroxycholesterol levels in the human aorta averaged 1.9 mg/g cholesterol (range 0.24-4.7 mg/g) (1), and our later measurements gave average intimal levels of 1.52 mg/g (range 0.094-7.6 mg/g) and average medial levels of 7.5 mg/g (range 0.15-83.5 mg/g) (2). These levels thus display a 100- to 500-fold increase over that in bovine brain (15 μg/g). In our previous examination of human brain we estimated that 26-hydroxycholesterol was not present at levels above 0.1-1 μg/whole human brain (1).

Species differences also obtain in the distribution of 24-hydroxycholesterol found in human (3,6,7,25) and equine (7,14,26) brain, but not in bovine or porcine brain (6,7,26). For comparison the absolute levels of 24-hydroxycholesterol are reported as 16 μg/g fresh human brain (6), 11 μg/g fresh equine brain (25,26), and 0.4-0.8 mg/g total equine brain sterols (14,25), a figure slightly lower than the level of 26-hydroxycholesterol in the human aorta but of the same order of magnitude.

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Dietary Regulation of Phosphatidic Acid Synthesis From Dihydroxyacetone Phosphate and Fatty Acid by Rat Liver Microsomes[1]

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ABSTRACT

Phosphatidic acid synthesis from dihydroxyacetone phosphate and 1-^{14}C-palmitate was studied in liver microsomes of rats maintained on a stock diet (5% fat), fasted for three days after being fed the stock diet, or given a high fat diet (15% fat) or a fat free diet for a week after being fed the stock diet. The amounts of phosphatidic acid synthesized per minute per milligram of microsomal protein in rats ingesting a stock diet or a fat free diet were at least twice the levels observed in rats either fasting or maintained on a high fat diet. Following fasting, realimentation with either a fat free or high fat diet returned the microsomal capacity for phosphatidic acid synthesis to approximately the same level, which was higher than that observed in rats maintained on a stock diet. Analogous results were observed when glyceraldehyde 3-phosphate was used as the glyceride-glycerol precursor, probably because microsomes convert glyceraldehyde 3-phosphate to dihydroxyacetone phosphate. These studies demonstrate that phosphatidic acid synthesis from dihydroxyacetone phosphate by particulate enzymes is influenced by diet.

INTRODUCTION

In recent years, numerous investigations have demonstrated the regulation of lipogenesis by dietary manipulations. These have involved mainly the several systems concerned with the biosynthesis of fatty acids. In spite of our detailed knowledge of the pathway for the synthesis of fatty acids, many of the complexities inherent in its metabolic control still remain to be elucidated (1). However, the activities of several of the soluble enzymes associated with fatty acid synthesis such as glucokinase (2), glucose 6-phosphate dehydrogenase (3,4), citrate cleavage enzyme (5,6), acetyl-CoA carboxylase (7,8) and fatty acid synthetase (3,9,10) have been shown to be reduced in mammalian liver when an animal is fasted. Subsequent feeding restores the activities of these enzymes to normal levels. In some cases where the animal is refed a fat free diet, these levels even exceed the normal (3,11). Recent studies using purified enzyme preparations have shown an adaptive synthesis of fatty acid synthetase (12) and independent factors regulating the rates of synthesis and degradation of acetyl-CoA carboxylase (13).

Our knowledge of the dietary adaptation of the particulate enzymic activities for the incorporation of fatty acids into glycerides is sparse. The results of our experiments demonstrate that the microsomal conversion of dihydroxyacetone phosphate (DHAP) to phosphatidic acid is subject to dietary regulation.

MATERIALS AND METHODS

The dimethyl ketal of dihydroxyacetone phosphate and the dimethyl acetal of DL-gly-

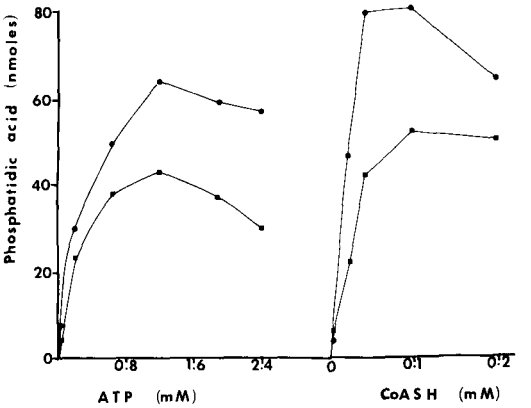

FIG. 1. Effect of varying cofactor and substrate concentrations on phosphatidic acid synthesis from DHAP (●) or DL-GAP (■) by washed rat liver microsomes. The standard incubation mixture, with methods of isolation and quantitation, have been previously described (16). Thirty min incubations were performed using 1-2 mg microsomal protein. The concentrations of ATP, CoASH, NADH, palmitate or triose phosphates were varied, keeping the remaining constituents constant, as indicated in the graphs. Different microsomal preparations were used for each experiment.

[1]Presented at the ISF-AOCS Meeting, September 1970.

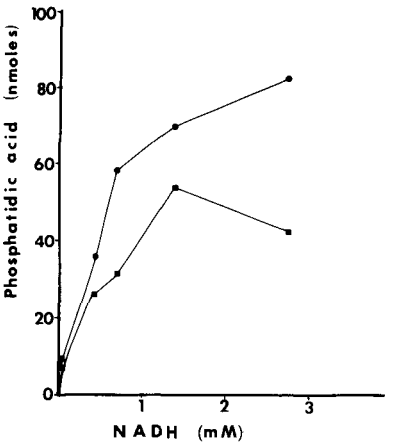

FIG. 2. See legend for Figure 1.

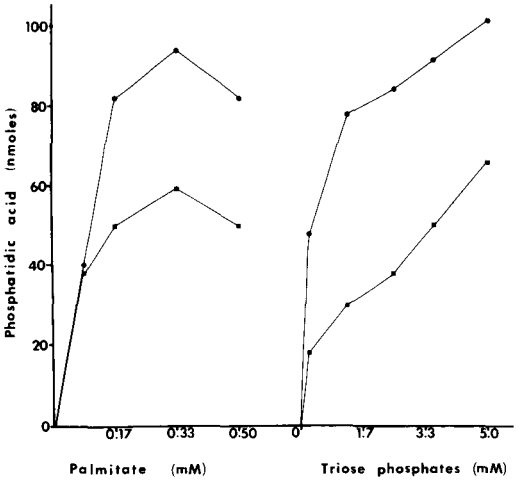

FIG. 3. See legend for Figure 1.

ceraldehyde 3-phosphate were purchased from Sigma Chemical Co. The free carbonyl compounds were regenerated by hydrolysis with Dowex 50-X4,H^+ and converted to their potassium salts using $KHCO_3$. 1-^{14}C-Palmitate from Volk Radiochemical Co. was used after purification (>99%) by thin layer chromatography. Phosphatidic acid used as standard was obtained from Pierce Chemical Co.

Male rats (Cheek-Jones) weighing 150-200 g were maintained on a stock ration (Purina Rat Chow, 5% fat), or a fat free diet (Nutritional Biochemicals), or a 15% fat diet (fat free diet plus safflower oil) for a week. Rat livers were removed immediately after killing the animals, and washed in ice cold 0.25 M sucrose. All subsequent operations were carried out at 0-5 C. Liver homogenates were prepared in 2 vol of 0.25 M sucrose using a loose fitting Potter Elvehjem homogenizer. The homogenate was centrifuged at 1,000 x *g* for 15 min and the pellet of whole cells, nuclei and cell debris was discarded. The 1,000 x *g* supernatant was centrifuged at 15,000 x *g* for 15 min to remove the mitochondria and lysosomes. The supernatant was siphoned carefully so as not to disturb the pellet, leaving 2-3 ml supernatant above it. The supernatant was then centrifuged at 104,000 x *g* for an hour. The microsomal pellet was suspended in 0.25 M sucrose in a volume corresponding to that of the original homogenate and centrifuged at 104,000 x *g* for 1 hr. Washed microsomes were suspended in a volume of 0.25 M sucrose corresponding to one half the volume of the original homogenate.

Incubations were carried out as described in the tables and terminated by adding 5 ml chloroform-methanol (2:1 v/v). After acidification with 0.2 ml 2N HCl, glycerides and phosphoglycerides were isolated and quantitated by procedures described by Johnston et al. (14). Protein determinations were carried out as described by Lowry et al. (15).

RESULTS AND DISCUSSION

Optimal Conditions for Phosphatidic Acid Synthesis From Triose Phosphates

Previous studies have shown that washed rat liver microsomes convert DHAP or glyceraldehyde 3-phosphate (GAP) to phosphatidic acid in the presence of fatty acid, ATP, CoASH and NADH (16). Several preliminary investigations were carried out to establish the optimum con-

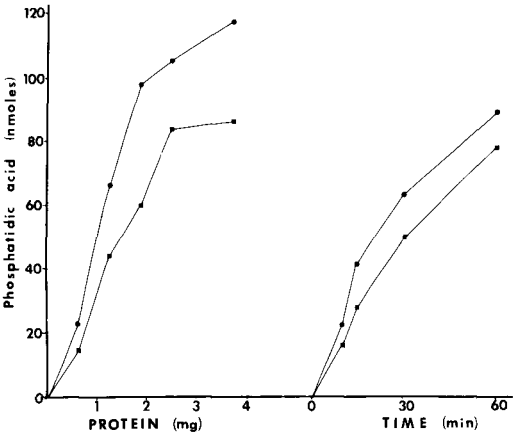

FIG. 4. Effect of microsomal protein and time of incubation on the synthesis of phosphatidic acid from triose phosphates (DHAP ●; DL-GAP ■). Conditions for incubation with methods of isolation and quantitation, are those previously described (16). Incubations were performed for 30 min varying the microsomal protein or keeping the microsomal protein constant at 1.2 mg with varying time intervals. Different microsomal preparations were used for each experiment.

TABLE I

Phosphatidic Acid Synthesis From Triose Phosphate by Hepatic Microsomes of Rats Under Different Dietary Conditions, Ad Lib[a]

Dietary status	Glyceride-glycerol precursor[b], mean ± st dev	
	DHAP	DL-GAP
Fasting	0.72 ± 0.22	0.54 ± 0.25
15% Fat diet	0.86 ± 0.18	0.42 ± 0.21
Stock diet	1.39 ± 0.21	1.11 ± 0.26
Fat free diet	2.15 ± 0.58	1.91 ± 0.36

[a]A group of rats was fasted for three days with free access to water. Another group which had been fed a stock diet was maintained for a week on a 15% fat diet. Yet another group fed a stock diet was maintained for a week on a fat free diet. There were six rats in each group. The reaction mixture contained potassium phosphate buffer (pH 7.4, 133 mM), ATP (1.3 mM), CoASH (0.06 mM), NADH (1.3 mM), GSH (3.3 mM), KF (4 mM), $MgCl_2$ (3.3 mM), DHAP (2.5 mM) or DL-GAP (5 mM), potassium-1-^{14}C-palmitate (0.3 mM), crystalline bovine plasma albumin (3 mg) and washed rat liver microsomes (1-1.2 mg) in a total volume of 1.5 ml. The reaction was terminated by the addition of 5 ml chloroform-methanol (2:1 v/v) after 30 min at 37 C in a Dubnoff shaker. Lipids were isolated and quantitated as described earlier (14). The nmoles of phosphatidic acid synthesized include this lipid observed at the end of the incubation period as well as that converted to di- and triglyceride.

[b]Nmoles of phosphatidic acid synthesized per minute per milligram.

centrations of cofactors and substrates for evaluation of the microsomal activities for the synthesis of phosphatidic acid from triose phosphates. The results of these studies are summarized in Figures 1–4. Phosphatidic acid synthesis is dependent on the presence of ATP, CoASH, NADH and triose phosphates since the exclusion from the incubation mixture of any one of these resulted in meager synthesis. Optimum synthesis of phosphatidic acid was obtained with ATP (1.3 mM), CoASH (0.06 mM), NADH (1.3 mM) and palmitate (0.3 mM). With similar concentrations, DHAP yielded significantly higher amounts of phosphatidic acid than DL-GAP presumably because only D-GAP is utilized for this process. However, even when DL-GAP was used in twice the molar concentration, it produced less phosphatidic acid than DHAP. This may be because DHAP is the direct acyl acceptor and GAP must be converted to DHAP by the microsomal triose phosphate isomerase prior to glyceride synthesis (16). Phosphatidic acid synthesis was proportional to the concentration of microsomal protein over a wide range. It increased in a fairly linear manner during a 60 min incubation period. With large amounts of microsomal protein, the presence of albumin in the reaction mixture did not exhibit any effect on phosphatidic acid synthesis. In its absence, presumably due to the destruction of the enzymic activity by the detergent action of potassium palmitate, meager and varied synthesis was observed with a small amount of microsomal protein. However, its presence in the reaction mixture with reduced amounts of microsomal protein yielded reproducible synthesis.

Effect of Dietary Status on the Conversion of Triose Phosphates to Phosphatidic Acid by Hepatic Microsomes

The amount of phosphatidic acid synthesized from triose phosphates per minute per milligram of microsomal protein of livers of rats fasted for three days, fed a 15% fat or a fat free diet for one week, or a stock diet, are given in Table I. The amount of phosphatidic acid produced was least with microsomes from fasted rats and those fed a 15% fat diet. About twice that amount of the lipid was produced when microsomes from livers of rats fed a stock diet were used. Even higher amounts of phosphatidic acid per minute per milligram of microsomal protein were produced when livers of rats fed a fat free diet were employed. Theoretically, a lower rate of conversion of fatty acid to acyl-CoA derivatives by hepatic microsomes of fasted rats or those fed a 15% fat diet could account for reduced phosphatidic acid synthesis. However, this is not the case since when glycerol 3-phosphate (GP) was used as the acyl acceptor, similar amounts of phosphatidic acid were synthesized per minute per milligram of microsomal protein of livers of fasted, 15% fat fed, and stock diet fed rats (unpublished data).

Enhanced hepatic acyl-CoA synthetase (17) and elevated levels of long chain acyl-CoA derivatives in liver (18,19) have been observed as a result of fasting. Thus the low level of phosphatidic acid synthesis in livers of fasting animals cannot be due to the limitation in amounts of either acyl-CoA derivatives or the enzyme which produces them. Hence, the decreased enzymic activity for the conversion of triose phosphates to phosphatidic acid rather than that for the activation of fatty acid must be responsible for the observed diminution in the synthesis of this lipid by the liver microsomes of these animals.

Previous investigations have shown that the DNA content of liver is not altered by fasting an animal (20-22). This constant DNA content of cells would indicate that the number of cells

TABLE II

Phosphatidic Acid Synthesis From Triose Phosphate by Hepatic Microsomes Following Refeeding After Fasting[a]

Dietary status	Number of animals	Glyceride-glycerol precursor[b], mean ± st dev	
		DHAP	DL-GAP
Fasting	6	0.72 ± 0.22	0.54 ± 0.25
Fat free diet	5	2.65 ± 0.56	2.05 ± 0.75
15% Fat diet	6	2.51 ± 0.38	1.76 ± 0.40

[a]Rats were fasted for three days with free access to water. Two groups of fasted rats were fed either a fat free diet or a 15% fat diet for three days before killing to determine the hepatic microsomal capacity for producing phosphatidic acid from triose phosphates. The reaction mixture contained potassium phosphate buffer (pH 7.4, 133 mM), ATP (1.3 mM), CoASH (0.06 mM), NADH (1.3 mM), GSH (3.3 mM), KF (4 mM), $MgCl_2$ (3.3 mM), DHAP (2.5 mM) or DL-GAP (5 mM), potassium-1-^{14}C-palmitate (0.3 mM), crystalline bovine plasma albumin (3 mg) and washed rat liver microsomes (1-1.2 mg) in a total volume of 1.5 ml. The reaction was terminated by the addition of 5 ml chloroform-methanol (2:1 v/v) after 30 min at 37 C in a Dubnoff shaker. Lipids were isolated and quantitated as described earlier (14). The nmoles of phosphatidic acid synthesized include this lipid observed at the end of the incubation period as well as that converted to di- and triglyceride.

[b]Nmoles of phosphatidic acid synthesized per minute, per milligram of microsomal protein.

in the liver is also not affected. However, a 40-50% decrease in the weight of liver was observed in rats after fasting three days as compared to those fed a stock ration. Furthermore, a similar decrease in the microsomal protein was also observed. This would be expected since the half life of microsomal protein has been reported to be about three days (23,24). Since the amount of phosphatidic acid synthesized from triose phosphates per milligram of microsomal protein is also reduced by half in fasting rats as compared to those on a stock diet, it is apparent that the liver cell in the fed animal is at least four times as active as that in the fasting. In addition, these results would suggest that the rate of degradation of the rate-limiting enzyme for phosphatidic acid synthesis from triose phosphate during the course of fasting may be at least twice that of the total microsomal proteins.

Subsequent to fasting, realimentation with either a fat free diet or a 15% fat diet for three days exhibited similar effects. These results are given in Table II. Phosphatidic acid synthesized per minute per milligram of microsomal protein increased at least three-fold. Since the weight of the liver and total microsomal protein is increased after refeeding, the liver cells are synthesizing these proteins at a rapid rate. Apparently 15% fat in the diet does not affect the rate of synthesis of the enzymes involved in phosphatidic acid synthesis from triose phosphates and fatty acid. Since a reduction of capacity of the microsomes to fasting level was observed after a week on this diet (Table I) the fat may in some manner enhance the rate of degradation of the rate-limiting enzyme in this process.

Several steps are involved in the biosynthesis of phosphatidic acid from DHAP and fatty acids. These consist of the activation of fatty acid, acylation of DHAP, reduction of acyl-DHAP and acylation of lysophosphatidic acid (25-27). The activity of one or all of the enzymes involved in the sequence may be responsible for the dietary response to lipid synthesis by microsomes. An evaluation of the individual enzyme activities would be required to pinpoint the regulatory step in the sequence. However, some possibilities exist which offer a helpful guide for future investigations. Previous experiments with rat liver (16) or hamster intestinal mucosa (28) have shown that unless acyl-DHAP reductase is functional, as when reduced pyridine nucleotide is available, acylation of DHAP occurs to only a small extent. This would suggest that acyl-DHAP reductase may be of regulatory importance in the synthesis of phosphatidic acid from triose phosphates. This is the only enzyme that is not needed for phosphatidic acid synthesis by the GP pathway. The other enzymes, namely glycerol 3-phosphate acyltransferase and lysophosphatidate acyltransferase may be shared by either the GP or DHAP pathways. These acyltransferases may not be subject to dietary regulation since similar amounts of phosphatidic acid per minute per milligram of microsomal protein from livers of fasting rats and those fed a stock diet were obtained when GP was used as the glyceride-glycerol precursor (unpublished data).

A determination of the acyl-DHAP reductase activity of microsomes has been difficult because of several factors. As shown earlier with guinea pig liver microsomes (26), rat liver microsomes also do not reduce the added acyl-DHAP to lysophosphatidic acid (16). In addition, acyl-DHAP is cleaved to acyl-DHA, DHAP and fatty acid by microsomal hydrolases (16). It is likely that the acyl-DHAP reductase utilizes the membrane-bound biosynthesized compound as substrate rather than that added as an aqueous suspension, as has been observed with the several other enzymes involved in lipid metabolism (14,29,30).

Numerous investigators have reported dietary modulation in the systems of fatty acid synthesis in the soluble fraction of liver cells. The data presented in this paper suggest the possibility of similar changes in the hepatic microsomal capacity to convert fatty acid and triose phosphates to phosphatidic acid. It can be concluded that concomitant adaptive changes in both the soluble and particulate fractions of the cell for the synthesis of fatty acid and its conversion to glyceride are involved in the dietary regulation of lipogenesis.

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Glycerides of *Limnanthes douglasii* Seed Oil

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ABSTRACT

Brockerhoff-type procedures were used to determine the amounts of each acyl group at each glyceride position of *Limnanthes douglasii* seed oil. During the course of the analyses, small quantities of three acids isomeric with those previously found in the oil were identified by their ozonolysis products and their gas-liquid chromatographic (GLC) behavior. The newly discovered constituents of the oil were 3-octadecenoic acid (0.1%), 5-octadecenoic acid (0.9%) and 11-eicosenoic acid (3%). The saturated acids and those with ω-9-unsaturation are esterified most often to β-glyceride positions in *Limnanthes* seed, while the acids with Δ5-unsaturation occur generally at the outer glyceride positions. Although the Δ5-unsaturated acids as a group exhibited no obvious preference for one outer position over the other, individual acids were unequally distributed between the 1- and 3-*sn*-glycerol positions. The probabilities of occurrence of the various triglycerides were calculated from the stereospecific analysis data by assuming a 1-random,2-random,3-random distribution of the acyl groups. The calculations are in agreement with the composition of the whole oil, as determined by GLC.

INTRODUCTION

More than 90% of the acyl groups in *Limnanthes douglasii* seed oil are represented by four entities, namely, *cis*-5-eicosenoate, *cis*-5-docosenoate, *cis*-13-docosenoate and *cis*-5,*cis*-13-docosadienoate (1-3). The remainder of the oil is composed of the common acyl groups, such as palmitate, stearate, oleate, linoleate, linolenate and small amounts of several other acyl moieties. Three minor acids, which are isomeric with the previously described acids, have now been identified by gas liquid chromatography (GLC) and by their ozonolysis products.

Although a host of oils have been hydrolyzed with pancreatic lipase to determine the acids esterified to their α- and β-glyceride positions, only a few triglycerides of natural origin have been subjected to a complete stereospecific analysis (4-13). The more common acyl groups in these oils are distributed among all three glyceride positions with oleate and linoleate esterified most frequently to β-glyceride positions. In some of the oils, which have unusual acyl groups, the exceptional acylate has been at only one glyceride position. The estolide groups of *Sapium sebriferum* (10), the acetates of *Euonymus verrucosus* (11) and of *Impatiens edgeworthii* (12), and the butyrate and hexanoate of two mammalian milk fats (8) are esterified exclusively to the 3-*sn*-glycerol hydroxyl (14). On the other hand, the trihydroxyoctadecenoates of *Chamaepeuce afra* (15) are attached exclusively to β-glyceride positions.

A program has been underway at the Northern Laboratory for several years to identify new and potentially useful fatty acids in seed oils (16). One of the seed oils investigated, which contains potentially useful and unusual acyl groups, is that of *L. douglasii* (1-3,17,18). The nonstereospecific intraglyceride distribution of a number of these new acids has been reported (19-22). With the advent of suitable procedures for stereospecific analyses, the research reported in this paper seemed a logical extension of the earlier work. Brockerhoff's (23,24) procedures, as modified by Christie and Moore (4), were used to acquire stereospecific data for the glycerides of *L. douglasii* seed oil. That is, the triglycerides were reacted with ethyl magnesium bromide to generate diglycerides, which were converted to phosphatidyl phenols. The latter were treated with the phospholipases of cobra venom. Only acyl groups bonded to the 2-position of the 1,2-diacyl-3-phenylphosphoryl-*sn*-glycerols (25) or to the 1-position of the 1,3-diacyl-2-phenylphosphoryl-*sn*-glycerols (26) are cleaved by phospholipase A (E.C. 3.1.1.4) of the venom.

EXPERIMENTAL PROCEDURES

General

Freshly ground *L. douglasii* seed was steeped twice in petroleum ether (bp 40-60 C) for 24 hr. Evaporation of the solvent with a stream of nitrogen on a warm water bath left a green oil in amounts approximating 30% of the seed (18). Column chromatography of the crude oil

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TABLE I

Analysis of *Limnanthes douglasii* Seed Oil

CN of glycerides	GLC response	Adjustment factors[a]	Glyceride composition[b]	Calculated composition[c]
52	0.7	0.9	0.3	2×10^{-3}
54	1.9	1.00	1.0	3×10^{-2}
56	4.0	1.06	2.3	0.6
58	8.8	1.12	5.1	3.9
60	34.5	1.44	25.1	25.8
62	38.7	2.32	44.0	43.3
64	10.8	3.99	20.5	22.1
66	0.5	6.57	1.5	3.3

[a]From a mixture containing known amounts of tristearin, triarachidin and tribehenin, adjustment factors for carbon numbers (CN) 60 and 66 were experimentally determined relative to CN 54 (1.00). The other factors were interpolated from a best fit curve drawn through these three data points.

[b]The peak areas were multiplied by the appropriate factors and the resulting "adjusted instrument response" used to compute the mole per cent of the glycerides by CN.

[c]The CN composition calculated for *L. douglasii* seed oil from a 1-random,2-random,3-random (13) combination of the fatty acids found esterified to each of the glyceride hydroxyls.

on CAB-Absorbosil with mixtures of hexane-ethyl ether (100:0 to 90:10, v/v) removed most of the color from the oil.

Methyl esters were prepared by refluxing the original oil, or the fatty acids or partial glycerides from the various enzymatic procedures, in 200 vol of 1 N methanolic sulfuric acid for 90 to 150 min. The resulting esters were recovered by diluting the alcoholic solutions with salt water and then extracting the aqueous phase several times with distilled hexane. The ester solution was dried over Linde molecular sieves, 3A, before evaporating the solvent with nitrogen.

Analytical GLC Procedures

Stainless steel columns, 1 mm (i.d.) X 3 m or a 1 mm (i.d.) X 6 m, packed with 100/120 mesh Chromosorb W-AWDMCS coated with LAC-2R-446 (3%) and thermostated at 180-190 C in a Hewlett-Packard 5750 chromatograph with a flame ionization detector, served for ester analyses. Confirmatory analyses were by GLC on a 1 mm (i.d.) X 3 m stainless steel column packed with 100/120 mesh Chromosorb W-AWDMCS coated with Apiezon L (5%) at 200 C. A 1 mm (i.d.) X 60 cm column of OV-1 (3%) on 100/120 mesh Gas-Chrom Q, programmed from 200 to 390 C at 4 C/min, was used for analyzing intact glycerides. The column which extended into the injection port made it possible to inject the samples directly on the column. The injection port temperature, while 310 to 330 C at the beginning of a glyceride analysis, increased to 370 to 380 C near the end of the temperature programming.

Peak areas were measured with an Infotronics integrator and were assumed to be proportional to the masses of the respective compounds, except for the glyceride peaks, which were adjusted as follows: The instrument response to a mixture of tristearin, triarachidin and tribehenin (1.04:1.00:1.03) was measured and adjustment factors were calculated for the C-60 and C-66 glycerides relative to tristearin. A best fit curve was drawn through the three data points from a graph of glyceride carbon numbers (CN) vs the respective adjustment factors. The adjustment factors for the other glycerides were interpolated from this curve. These values (Table I, column 3) are intermediate between those reported by other workers (27,28). The adjusted glyceride composition of the oil was computed by multiplying the peak areas by the appropriate adjustment factors to obtain adjusted mass ratios, which subsequently were used to compute the mole percentages of the various glycerides.

Preparative GLC Procedures

After obtaining an analytical chromatogram of the various methyl ester mixtures, they were fractionated by preparative GLC with a 4 mm (i.d.) X 3 m column packed with 100/120 mesh Gas Chrom Q, with a 3% coating of OV-1, into C-18, C-20 and C-22 esters. Because of the low levels of column substrate, no more than 5 mg of ester mixture could be resolved per pass. The fractions were collected by channeling the column effluent through a glass tube, which was loosely packed with Adsorbosil. The esters were eluted from the silica with chloroform and examined on the analytical columns to ascertain their purity. If esters of the designated

CN accounted for less than 95% of the respective fraction, it was rechromatographed on the preparative column.

Thin Layer Chromatographic Procedures

Thin layer chromatographic (TLC) analyses were carried out on 0.25 mm layers of Silica Gel G with the following as solvents: Solvent A, petroleum ether-ethyl ether (1:1); Solvent B, chloroform-methanol-ammonia (80:20:2); and Solvent C, petroleum ether-ethyl ether-acetic acid (50:50:1). The spots on the developed chromatograms were made visible by staining with iodine, or by charring with a sulfuric acid-chromic oxide reagent at 125 C, or both. Preparative chromatograms utilized 0.25, 1.00 or 2.00 mm layers of silica gel, depending on the amount of material to be separated. The components were located by viewing under an UV lamp, after the layers of silica were sprayed with dichlorofluorescein. The partial glycerides and fatty acids were washed from the support with ethyl ether, and the phospholipids were eluted with chloroform-methanol (1:1).

Preparation of Diacylglycerols

The α,β- and α,α'-diacylglycerols required for the stereospecific analyses were prepared from the triglycerides by means of their reaction with ethyl magnesium bromide following Christie and Moore's (4) modification of Brockerhoff's (24) procedures. The diacylglycerols were separated from the other reaction products by preparative TLC with Solvent A.

Preparation of Phosphatidyl Phenols

The diacylglycerols (5 to 150 mg) were dissolved in 15 to 20 vol of pyridine, which had been distilled from barium oxide, and were mixed with 6 to 10 equivalents of vacuum-distilled phenyl dichlorophosphate. The air in the reaction vessel was replaced with dry nitrogen. After the mixture was kept at room temperature for 16 to 20 hr, the unreacted phosphoryl chloride was destroyed by adding aqueous pyridine while cooling. The reaction mixture was partitioned in a chloroform-methanol-water (9:8:7) system; the chloroform layer was separated; and the aqueous phase was reextracted with another portion of chloroform. Triethyl amine (1 ml) was added to the combined chloroform extracts, and the mixture was concentrated on a rotary evaporator. The synthetic phospholipids were purified by preparative TLC on 0.25 mm layers of Silica Gel H with Solvent B.

Phospholipase A Hydrolyses

Approximately 20 mg of *Ophiophagus hannah* vanom (Ross Allen Reptile Institute, Silver Springs, Florida) was dissolved in 3 to 4 ml of 0.5 M Tris buffer for each 50 mg of phosphatides to be hydrolyzed. The buffer solution, pH=7.5, was 2.0×10^{-3} M in calcium chloride. The venom solution was extracted with several portions of ethyl ether, 2 to 4 ml each, before being used for phosphatide hydrolysis. When the venom solutions were not extracted, the final products were contaminated with acids foreign to the *Limnanthes* oil.

The phosphatidyl phenols were dissolved in ethyl ether (50 mg/10 ml of solution) and were vigorously mixed with the washed venom solution by means of a magnetic spin bar in a small vial. Samples of the ether phase were removed from time to time and chromatographed in Solvent B to determine the extent of hydrolysis. Usually no further changes in the reaction mixture were evident after 6 hr. The lipid materials were recovered by diluting the mixture with salt water and extracting the aqueous phase with several portions of ether. The fatty acids were separated from the lysophosphatides and unreacted phosphatides by chromatography on 0.25 mm layers of silica with Solvent C. The phosphatides, which remained near the origin, were recovered from the silica and rechromatographed on another layer of silica in Solvent B to separate the two types. The fatty acids were eluted from the silica and converted to methyl esters.

Pancreatic Lipase Hydrolyses

Pancreatic lipase (E.C. 3.1.1.3) catalyzed hydrolyses of the seed oil were patterned after the procedure given by Luddy et al. (29), except that the mixture was agitated with an ultrasonic probe (30) rather than an amalgamator. After a reaction time of 5 min, the mixture was diluted with salt water and was extracted several times with ether. The combined ether extracts were dried over Linde sieves, 3A. The dried solution was concentrated with a stream of nitrogen, and the free acids were esterified with an excess of ethereal diazomethane. The monoglycerides were then separated from the other lipid products by TLC with Solvent A. Methyl esters were prepared from the monoglycerides as described earlier.

Ozonolysis

The ester mixtures were ozonized and the products analyzed by procedures and equipment similar to those described by Keiman et al. (31), except for the addition of an excess of ozone to ensure complete ozonolysis of all the double bonds in the mixtures and the use of hexane rather than methylene chloride as solvent for the ozonolysis (32). The ester fractions, usually less than 2 mg, were dissolved in

TABLE II

Stereospecific Distribution of Acids in *L. douglasii* Seed Oil

	C-1		C-2			C-2(venom)			C-3		Whole oil			Proportions at		
Acid	GLC[a]	O_3[b]	GLC[c]	O_3[b]	C-2(10)[d]	GLC[e]	O_3[b]	C-2+3[f]	GLC[g]	O_3[b]	GLC[h]	O_3[b]	C-1+2+3[i]	C-1[j]	C-2[j]	C-3[j]
10:0	—		—		—	—		—	—		Trace		—			
12:0	—		—		—	—		0.1	—		Trace		—			
14:0	Trace		0.3		Trace	Trace		0.1	Trace		0.2		0.1			
16:0	0.2		0.4		0.5	0.6		0.1	Trace		0.2		0.2	27	67	7
16:1	0.2		0.2		0.1	0.1		Trace	Trace		0.3		0.1	57	28	14
18:0	0.3		0.5		0.4	0.5		0.3	0.07		0.2		0.3	39	52	9
18:1 Δ3	0.03		0.1	0.1					0.1	0.3	0.06	0.06	0.1			
18:1 Δ5	1.1		4.0	0.4	2.7	3.2		2.4	0.3	0.4	0.8	0.9	0.6	65	18	18
18:1 Δ9	1.4			3.5					0.7	0.5	1.5	1.5	1.2	31	53	15
18:2	0.2		0.4		0.4	0.5		1.0	0.06		0.3		0.2	30	61	9
18:3	0.2		0.6		0.4	0.5		1.0	0.14		0.3		0.3	27	54	19
19:0 (?)	0.05		0.1		Trace	Trace		—	—		0.1		Trace			
20:0	1.2		1.3		1.4	0.7		1.2	0.7		0.7		1.0	36	42	21
20:1 Δ5	70.9[k]	72	38.5	37	42.3	47.1	46	58.5	63.1	62	65.2	63	60	42	22	36
20:1 Δ11	3.4[k]	3		2			1			1		3	2	43	43	14
20:2	0.4		Trace		Trace	Trace		Trace	Trace		0.4		Trace			
22:0	0.3		Trace		Trace	0.2		—	—		Trace		0.1			
22:1 Δ5	3.2[k]	3	45.0	2	43.9	40.0	4	28.3	18.0	4	18.4	3	4	33	22	44
22:1 Δ13	5.5[k]	5		43			29			16		14	17	8	67	25
22:2	11.2	12	9.1	10	7.9	6.9	14	7.0	12.7	11	11.1	13	12	35	25	40
22:3	—		—		—	0.2		—	—		0.5		—			
22 (?)	—		—		—	0.4		—	—		0.1		—			
24:0	—		—		—	—		—	—		0.2		—			

[a]Mole percentages calculated from gas liquid chromatographic (GLC) analyses of methyl esters derived from the 1-acyl-3-phenylphosphoryl-*sn*-glycerols.

[b]Mole percentages calculated on the basis of the products obtained from ozonolysis of the respective methyl ester mixtures.

[c]Mole percentages calculated from GLC analysis of methyl esters derived from the monoglycerides formed by the pancreatic lipolysis procedure of Luddy et al. (29).

[d]Mole percentages calculated from GLC analysis of methyl esters derived from monoglycerides which were obtained from a pancreatic lipolysis of the oil with 10 times the usual amount of lipase.

[e]Mole percentages calculated from GLC analysis of methyl esters of fatty acids liberated by the phospholipase A-catalyzed hydrolysis of the 1,2-diacyl-3-phenylphosphoryl-*sn*-glycerols.

[f]Mole percentages calculated from GLC analysis of methyl esters derived from the phosphatidyl phenols that did not react with phospholipase A, presumably 2,3-diacyl-1-phenylphosphoryl-*sn*-glycerols.

[g]Mole percentages calculated from GLC analysis of methyl esters derived from 3-acyl-2-phenylphosphoryl-*sn*-glycerols.

[h]Mole percentages calculated from GLC analysis of methyl esters obtained by means of an acid-catalyzed transmethylation of the seed oil.

[i]Mole percentages calculated by averaging the respective values in columns C-1, C-2(10) and C-3.

[j]Percentage of each acyl group that is esterified to each glyceride position, e.g., the proportion at C-1 = [(C-1)/(amount in seed oil) x 3] 100.

[k]These values were obtained by reconstructing the partially resolved GLC peaks with an analog computer (37).

2 ml of purified hexane (Burdick and Jackson Laboratories, Inc., distilled in glass quality) and cooled to dry ice temperatures while under a moderate vacuum to prevent condensation of moisture in the reaction vessel. After release of the vacuum, the ozone-oxygen mixture was passed through the solution for 30 to 45 sec. Excess ozone was removed by vigorously shaking the solution while it was being evacuated once more and then warmed to room temperature. After the hexane was evaporated with nitrogen, the ozonides were dissolved in a small amount of methylene chloride, a good solvent for the excess of triphenylphosphine used to reduce the ozonides. The resulting solutions were analyzed as described (31).

RESULTS

Ester Analysis

In addition to components reported previously (1-3), the 20:1 Δ11 and two C-18 esters that had not been detected before in esters derived from *L. douglasii* seed oil were identified by their GLC behavior on LAC-2R-446 columns and their ozonolysis products. One C-18 ester had an equivalent chain length (ECL) (33) of 18.55, and another an ECL of 18.21 (vs 18.31 for methyl oleate). Kleiman et al. (34) reported an ECL of 18.6 for methyl *trans*-3-octadecenoate. The methyl *cis*-5-octadecenoate derived from *Carlina* seed oil (35) has an ECL which is less than that of methyl oleate by 0.10 (G.F. Spencer, private communication). Gunstone et al. (36) found a similar relationship of ECL's for the three isomeric octadecenoates on a DEGS column. The ECL's of these two methyl esters from *Limnanthes* suggest that they are methyl 3-octadecenoate and methyl 5-octadecenoate, respectively. The quantities of the various 18:1 esters were computed from their GLC peak areas and ozonolysis fragments (Table II). Cleavage products were identified by their ECL's on both the Apiezon and R-446 columns (31). No attempt was made to determine the geometric configurations of the octadecenoates.

Only partial separation was achieved of the esters of the two C-20 monoenes and likewise of those of the two C-22 monoenes on the 6 m R-446 column. The quantitative data given in column 2 of Table II for these four acids is based on estimates made by analysis of the incompletely resolved GLC peaks with an analog computer (37). For both the 20:1 pair and the 22:1 pair, the results are in good agreement with relative amounts calculated from ozonolysis products (Table II, column 3). Consequently, the values from ozonolysis results were considered sufficient for the individual members of these pairs in other fractions for which data are reported in the Table.

Stereospecific Analyses

L. douglasii oil is only slowly hydrolyzed to monoglycerides (25%) and diglycerides (16%) by pancreatic lipase under conditions that completely hydrolyze soybean oil to monoglycerides and free fatty acids. However, the hydrolysis of the seed oil with 10 times the amount of pancreatic lipase suspension recommended by Luddy et al. (29) for 5 min resulted in complete disappearance of triglycerides, according to TLC of the reaction mixture. The resulting monoglycerides were transmethylated with acidic methanol and were analyzed by GLC (Table II, "C-2(10)").

The reaction of the seed oil with ethyl magnesium bromide resulted in a 19% yield of diglycerides (4). After separating the diglycerides from the other reaction products by preparative TLC, they were phosphorylated with phenyl dichlorophosphate and separated into an α,α'-diacyl-β-phenylphosphoryl glycerols (α,α'-DAP) and α,β-diacyl-α'-phenylphosphorylglycerols (α,β-DAP) in a 1:2 ratio. The slower migrating α,β-DAP fraction (R_f 0.64) was contaminated with small amounts of the α,α'-DAP (R_f 0.72) and was rechromatographed to improve its purity.

The reaction of the α,α'-DAP with phospholipase A was essentially complete in 6 hr. The resulting 3-acyl-2-phenylphosphoryl-*sn*-glycerols were separated from the fatty acids by TLC and transmethylated. The complete analyses of the esters derived from C-3 are summarized in Table II.

According to TLC, the extent of the phospholipase A-catalyzed hydrolysis of α,β-DAP increased up to 3.5 hr and remained constant thereafter. The reaction mixture was separated into unreacted α,β-DAP, lysophosphatides and fatty acids. The complete analysis of the methyl esters of the fatty acids are summarized in Table II, column "C-2(venom)." The esters derived from the 1-acyl-3-phenylphosphoryl-*sn*-glycerols are listed under "C-1" of Table II. The unreacted α,β-DAP, presumably 2,3-diacyl-1-phenylphosphoryl-*sn*-glycerols, were also transmethylated and examined by GLC (Table II, column "C-2+3").

DISCUSSION

During the course of this stereospecific analysis, three acids isomeric with those reported a decade ago (1,2) were identified by their ECL's and ozonolysis products. These acids, 3-octadecenoic, 5-octadecenoic and

TABLE III

Most Probable Glycerides of *L. douglasii* Seed Oil by 1-Random,2-Random,3-Random Combination

Position 1	Position 2	Position 3	Mole %
20:1 Δ5	20:1 Δ5	20:1 Δ5	18.4
20:1 Δ5	22:1 Δ13	20:1 Δ5	18.3
20:1 Δ5	20:1 Δ5	22:1 Δ13	4.7
20:1 Δ5	22:1 Δ13	22:1 Δ13	4.6
20:1 Δ5	22:2 Δ5,Δ13	20:1 Δ5	3.6
20:1 Δ5	20:1 Δ5	22:2 Δ5,Δ13	3.2
20:1 Δ5	22:1 Δ13	22:2 Δ5,Δ13	3.2
22:2 Δ5,Δ13	20:1 Δ5	20:1 Δ5	2.9
22:2 Δ5,Δ13	22:1 Δ13	20:1 Δ5	2.9
20:1 Δ5	22:1 Δ5	20:1 Δ5	1.8
22:1 Δ13	20:1 Δ5	20:1 Δ5	1.4
22:1 Δ13	22:1 Δ13	20:1 Δ5	1.4
20:1 Δ5	18:1 Δ9	20:1 Δ5	1.3
20:1 Δ5	20:1 Δ5	22:1 Δ5	1.2
20:1 Δ5	22:1 Δ13	22:1 Δ5	1.2
20:1 Δ5	20:1 Δ11	20:1 Δ5	1.0

11-eicosenoic, together with the acids previously identified, suggest that at least two homologous series of acyl groups are synthesized by the maturing *Limnanthes* seed; i.e., a group with 5,6-unsaturation and a group with ω-9-unsaturation. Further, the major dienoic, if not other unidentified minor ones, is a member of both the 5,6-unsaturated and the ω-9-unsaturated series of acids. Such mixtures of acyl groups, i.e., synthesized by more than one desaturase system, occur in a significant number of seed fats that have been carefully analyzed (16).

In the analysis of triglycerides composed of the common, long chain acyl groups, pancreatic lipase has found widespread use for determination of the fatty acids esterified to the β-glyceride positions. However, if long reaction times are necessary for hydrolysis, this lipase has limited analytical value because the acyl groups may readily migrate from the β-glyceride positions to the outer glyceride positions (38). Because pancreatic lipolysis of *Limnanthes* seed oil is slow, diglycerides so obtained have limited usefulness for stereospecific analytical procedures (39). Fortunately the lipolysis reaction with a 10-fold increase in the amount of pancreatic lipase probably provided a representative sample of 2-monoglycerides for this study because no unreacted triglycerides remained and the short reaction time prevented any extensive group migration.

The slowness of the pancreatic lipase-catalyzed hydrolysis of *Limnanthes* triglycerides is most likely due to the presence of the Δ5 double bonds rather than the long chain length of the acyl groups in the oil. This conclusion is based on the following arguments: crambe oil, which has a high *cis*-13-docosenoate content, is hydrolyzed at a rate equal to or greater than that of soybean oil (40); *Limnanthes* oil does not crystallize above 1 C (16); because the oil was dispersed in the buffer before addition of the enzyme suspension, its slow hydrolysis rate apparently was not due to a failure to provide the required oil-water interface (41,42). Seed lipids with Δ3-unsaturated acyl groups (40) and whale oil, which contains long chain acyl groups with unsaturation near the carboxylate, i.e., 20:5 and 22:6 acids (43, and references cited therein), are only partially hydrolyzed by pancreatic lipase. Thus, the position of unsaturation in triglycerides has a more profound influence on the results of pancreatic lipolysis than either chain length or the lack of unsaturation, since the latter can be partially circumvented by the addition of an organic solvent (42).

As in most other seed oils that have been examined stereospecifically, each of the major fatty acids of the *Limnanthes* seed was found esterified to all three glyceride positions, although not in equal amounts. Examination of Table II shows that acids with ω-9-unsaturation, including 13-docosenoic (erucic) acid, are bonded most frequently to the 2-position, while those acids with Δ5-unsaturation are esterified most often to the 1- and 3-glyceride positions. The high frequency of erucate at the β-position is in contrast to its distribution in rapeseed and related oils (44,45).

The only simple, and at the present time, feasible approach to the glyceride structure of an oil from a stereospecific analysis of its fatty acid distribution is to assume a 1-random,2-random,3-random distribution (13) of the various acids. If such a combination is assumed, the acids of Table II may be combined into

more than 5100 different glycerides. With the aid of the computer we have found that only 251 of the possible combinations are probably above the 0.01% level. Those probable above the 1% level have been listed in Table III.

The totals of probably glycerides grouped by CN are listed in the last column of Table I and, in general, agree well with the experimentally determined values given in column 4 of the same table. The significantly higher amounts found that calculated for relatively low CN species suggest a deviation from the simple 1-random,2-random,3-random pattern in that the smaller acids are apparently combined preferentially into glycerides not containing C-20 and C-22 acids.

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The Isolation and Specificity of Alfalfa Lipoxygenase[1]

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ABSTRACT

Partial purification of the lipoxygenase from alfalfa seed was accomplished by fractionation of the protein with $(NH_4)_2SO_4$, phosphate, heavy metal salts and ultracentrifugation. About 24% of the original activity was recovered. The partially-purified alfalfa lipoxygenase enzyme was free of hydroperoxide-decomposing activity and was used to determine the positional specificity of linoleic acid oxidation by alfalfa lipoxygenase. Combined gas liquid chromatography-mass spectrometry was used to analyze known mixtures of 10- and 12-hydroxystearic acid derivatives and was satisfactory for the quantitative determination of the ratio of each component. This combination was used to analyze mixtures containing position isomers of hydroxy fatty acids without separation of each individual compound by other methods. Hydroperoxides produced from linoleic acid oxidation catalyzed by alfalfa lipoxygenase were converted by sodium borohydride reduction, catalytic hydrogenation and bis(trimethylsilyl)acetamide silylation to their corresponding trimethylsilyl ether esters and the positional distribution was studied. The 9- and 13-linoleate hydroperoxides produced by alfalfa lipoxygenase were in equal concentrations (50:50) whereas the distribution for soybean lipoxygenase was 70% 13- and 30% 9-hydroperoxides.

INTRODUCTION

Lipoxygenase (EC. 1.13.1.13) is an enzyme which acts specifically on polyunsaturated fatty acids containing a *cis, cis*-methylene-interrupted diene moiety (linoleic acid, linolenic acid, etc.) to form the *cis, trans* conjugated diene hydroperoxides. The enzyme is found primarily in plant seeds, particularly in legumes (1) and, to some extent, in animal tissues (2). The first crystalline lipoxygenase was prepared by Theorell et al. (3) from soybean seed. The properties of lipoxygenase were studied with this crystalline enzyme (4) as well as with enzymes from other sources (5-7).

Hamberg and Samuelsson (8) reported that the hydroperoxyoctadecadienoic acids produced from linoleic acid oxidation catalyzed by soybean lipoxygenase were 70% 13-hydroperoxyoctadecadienoic acid and 30% 9-hydroperoxyoctadecadienoic acid but later reported a ratio of 92% 13- and 8% 9-hydroperoxy (9). Dolev et al. (10) reported the production of 100% of the 13-isomer using crystalline soybean lipoxygenase. More recently Zimmerman and Vick (11) reported a large variability in the percentage of 9-isomer (6% to 30%) using flaxseed lipoxygenase.

Enzymes capable of catalyzing the decomposition of hydroperoxides have been reported in the crude extract of many oil seeds such as soybean (12), alfalfa (13), flax seed (14,15) and corn (16). The coexistence of lipoxygenase and the hydroperoxide-decomposing enzymes in the same extract caused some difficulty in the evaluation of lipoxygenase activity. The latter enzymes also posed potential problems in the determination of the products of lipoxygenase reaction. It has been reported that the hydroperoxide-destroying enzymes are heat labile (11-17).

The variation in the isomer distribution mentioned above could arise through selective destruction of specific isomers by hydroperoxide-decomposing enzyme or through analytical error. We have developed an improved method of enzyme isolation and a reproducible method of product determination using combined gas liquid chromatography-mass spectrometry (GLC-MS). In our analysis the hydroperoxides produced by alfalfa lipoxygenase were analyzed by the GLC-MS method developed by Ryhage et al. (18). This consists of taking mass spectra at time intervals during the elution of the compounds from the GLC column.

MATERIALS AND METHODS

Sources of Materials

Alfalfa seeds of the Ranger variety were purchased from Agway Inc., State College, Pa. Pure linoleic acid (99%) and bis(trimethylsilyl)

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TABLE II

Specific Activities of Alfalfa Lipoxygenase During Three Stages of Purification

Fraction	Specific Activity (μmoles/min/mg) 1	2	3
Crude	9.4	14.6	16.3
1st $(NH_4)_2SO_4$·ppt	20.6	21.1	13.8
Phosphate-treated	38.7	21.7	25.0
Supernatant after Pb(OH)Ac and $BaAc_2$ ppt	37.7	27.4	16.6
2nd $(NH_4)_2SO_4$ ppt	206	208	162
Supernatant, centrifuged 2 hr at 5.94×10^4 x g	240	304	205

acetamide were purchased from Supelco Inc., Bellefonte, Pa., and stored in the freezer (-20 C). Sodium borohydride was a product of the Metal Hydrides Inc., Beverly, Mass. Pure 12-hydroxystearic acid (99%) was purchased from Applied Science Lab., State College, Pa., and stored in the freezer (-20 C). The 10-hydroxystearic acid was a gift from Dr. Walter Niehaus of this department. Crystalline soybean lipoxygenase was purchased from Worthington Biochemical Co., Freehole, N.Y., and stored in the freezer (-20 C).

Enzyme Assay

Lipoxygenase and hydroperoxide-decomposing enzyme assays were performed on a Cary Model 14 spectrophotometer. Lipoxygenase was assayed by measuring the increase of absorption of the conjugated diene hydroperoxide at 234 nm (19). The substrate was prepared by combining 4 ml of H_2O, 30 μl of pure linoleic acid, 0.99 ml of 0.1 N NaOH solution and 15 μl of Tween 20. The mixture was shaken vigorously and diluted to 25 ml with water. The assay was conducted by following the increasing absorbance at 234 nm of a mixture of 0.7 ml of 0.1 M phosphate buffer, pH 6.8, 0.1 ml of substrate and 0.1 ml of oxygenated enzyme preparation in a 1 cm cuvette with a quartz spacer to give a 3 mm light path. The blank cuvette contained 0.8 ml of 0.1 M phosphate buffer, pH 6.8 and 0.1 ml of enzyme solution. The rate constants were calculated from the essentially linear portion of the absorbance curve.

Hydroperoxide-decomposing enzymes were assayed by measuring the rate of disappearance of absorption at 234 nm. The substrate, linoleic hydroperoxide, was prepared by reacting 9.6 mg of commercial soybean lipoxygenase with 30 μl linoleic acid in 50 ml of water containing 0.99 ml of 0.1 N NaOH solution and 15 μl of Tween 20, for 30 min at room temperature. The assay mixture contained 0.1 ml of the hydroperoxide solution diluted with 0.7 ml phosphate buffer, pH 6.8, 0.1 ml of enzyme preparation and was followed spectrophotometrically at 234 nm. 234 nm absorption.

Disc Gel Electrophoresis

Analytical dis gel electrophoresis was performed on the enzyme solution as described by Davis (20). A Shanden powder supply and Canal Industrial Co. reagents were used.

Different concentrations of enzymes were used on the acrylamide disc gel electrophoresis. Proteins were stained with 25 mg% Coomassie Brilliant Blue in 10% trichloroacetic acid (TCA). The starch-iodine staining method developed by Guss et al. (21) was used in the determination of active protein. After electrophoresis, the gel was soaked in substrate for 20 min, washed and then soaked in a potassium iodide (KI) solution. Starch was included in preparing the gel. The active proteins, after staining, appeared as a brown band.

Diethylaminoethyl Cellulose Chromatography

The ion exchange cellulose used in this study was Whatman DE-32 purchased from Scientifica Division of Reeve Angel. The powdered dry cellulose derivative was swollen and precycled according to the methods described by the manufacturer (22).

Product Preparation and Derivatization

The enzyme solution was oxygenated by sparging with O_2 for 1 min. Equal volumes (25 ml) of oxygenated enzyme and substrate were mixed and diluted with three volumes (75 ml) of 0.1 M phosphate buffer, pH 6.8. The mixture was shaken for 1/2 hr, then acidified by 6 N HCl to pH 2-3. The products were recovered by extracting three times with 200 ml ethyl

TABLE I

Lipoxygenase and Hydroperoxidase Activities During Enzyme Purification

Fraction	Total protein, mg	Lipoxygenase			Hydroperoxide decomposing enzymes	
		Total[a] activity, μmole/min	Specific[a] activity, μmoles/min/mg	Activity recovered[b], %	Total[a] activity, μmole/min	Specific[a] activity, μmoles/min/mg
Crude	1164	17,000	14.6	100	22,800	19.5
1st $(NH_4)_2SO_4$ ppt[c]	ND[d]	ND[d]	21.1[e]	ND[d]	ND[d]	27.2[e]
Phosphate treated[c]	77.3	17,000	21.7[e]	10[f]	850	11.0[e]
Supernatant after $Pb(OH)(CH_3COO)$ and $BA(CH_3COO)_2$ ppt	36.5	1000	27.4	6[f]	0	0
2nd $(NH_4)_2SO_4$ ppt	13.1	2700	207	16[f]	0	0
Supernatant, centrifuged 2 *hr at* $5.94 \times 10^4 \times g$	13.0	4100	304	24	0	0

[a]Total activities are expressed in μmoles of substrate being converted in the first minute of assay. Specific activities are expressed as μmoles of substrate being converted per minute per milligram of protein.

[b]The total activity in the crude extract was underestimated because of the presence of the hydroperoxidase, thus the per cent of recovery is the maximum value in each step.

[c]The supernatant solutions of these steps could not be assayed because of the high salt concentration. Neither enzyme was stable to dialysis.

[d]Not determined.

[e]The total protein and total activity were not measured. The specific activity was calculated from activity and protein content determined on solutions containing a small amount of precipitate from these steps dissolved in water.

[f]Assays of recoveries at these intermediate stages are not accurate because of the presence of inhibiting substances.

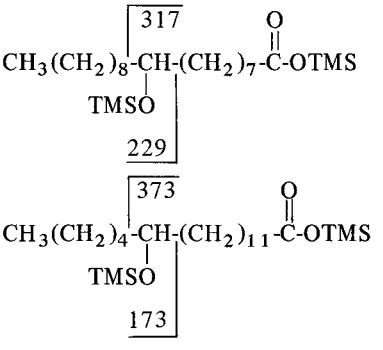
ether. The hydroperoxides were isolated on a preparative thin layer chromatography (TLC) plate, developed in *n*-heptane-ethyl ether-acetic acid, 50:50:1 (v/v).

The hydroperoxides, recovered from the silica gel by extraction with ethyl ether, were reduced by $NaBH_4$ and all double bonds were saturated by catalytic hydrogenation as described by Hamberg and Samuelsson (9). The resulting hydroxystearic acids were dried in a stream of N_2 and silylated with bis(trimethylsilyl)acetamide (23).

Gas Liquid Chromatography - Mass Spectrometry

The silylated ether-esters of hydroxystearic acids were identified on a Varian Aerograph, Model 1740, using 150 cm column of 1% OV-1 on celite, at 195 C. The 10- and 12-OTMS-TMS-stearates were used as standards. The derivatized fatty acids were analyzed on a LKB-9000 combined gas chromatograph-mass spectrometer equipped with a column of 1% SE-30 on celite. The proper GLC peaks were scanned on the mass spectrometer at intervals of 5 sec between m/e 275 and m/e 400. Fifteen to 20 scans were taken as the unresolved GLC peak came off the column. Intensities of peaks having m/e values 317 and 373 representing 9-OTMS and 13-OTMS-TMS-stearate, respectively, were then plotted versus time.

Since soybean lipoxygenase was reported as producing 30% 9- and 70% 13-linoleic hydroperoxide (8), the products of linoleic acid oxidation using commercial soybean lipoxygenase were also prepared and derivatized. The m/e values selected for silylated 9- and 13-hydroxystearic acids were 317 and 373 which result from cleavages as diagrammed below.



These m/e values are the major cleavages in the mass spectrum (23). The individual graphs were plotted and integrated by the weighed paper method (24). Subsequently, the ratio of each component in the mixture was calculated from these values. The same MS method was applied to the analysis of derivatized products of alfalfa lipoxygenase.

RESULTS

The method used in the partial purification of alfalfa lipoxygenase is diagrammed in Figure 1. During the purification procedure all centrifugation steps were performed at O C. The acetone powder was prepared at room temperature. After extraction with acetone the residue material was dried under vacuum and could be stored at -20 C without loss of activity for a period of eight weeks. Extraction of the acetone powder was achieved by stirring in acetate buffer for 3 hr at 10 C. The specific activity was improved 20-fold as the final enzyme preparation was achieved. The result of a representative isolation is shown in Table I. Specific activities of the stages of purification of three enzyme purifications are listed in Table II.

Diethylaminoethyl cellulose column chromatography was attempted on the acetone powder extracts with poor yields of active lipoxygenase. The enzyme remained bound to the column even though the hydroperoxide-decomposing enzyme activity could be eluted with 0.2 M NaCl solution. Lipoxygenase was not destroyed on the column since some lipoxygenase activity could be detected by passing an oxygenated linoleic acid solution through the column. The enzyme could not be eluted from the column by high salt concentrations at various pH's in the presence or absence of substrate.

Samples of 150 μg of soybean lipoxygenase and 200 μg of alfalfa lipoxygenase prepared as outlined in Figure 1, were analyzed by disc gel electrophoresis. The resulting gels (Fig. 2) were stained with starch-I_2 as developed by Guss et al. (21) and Coomassie Brilliant Blue.

The products of lipoxygenase were analyzed by reduction and silylation of the hydroperoxides, followed by GLC-MS. Standard quantitative mixtures of pure 10-hydroxystearic and pure 12-hydroxystearic acids were prepared and silylated. The mixture containing 54% 10-OTMS-TMS-stearate and 46% 12-OTMS-TMS-stearic was scanned on the mass spectrometer. Intensities of m/e 331 and 359 were measured at 5 sec intervals and plotted versus time. The areas under m/e 331 and m/e 359 were 57% and 43% respectively, which compare favorably with the original quantities of 10- and 12-hydroxystearic acids used.

The distribution of products using soybean lipoxygenase determined by this method was

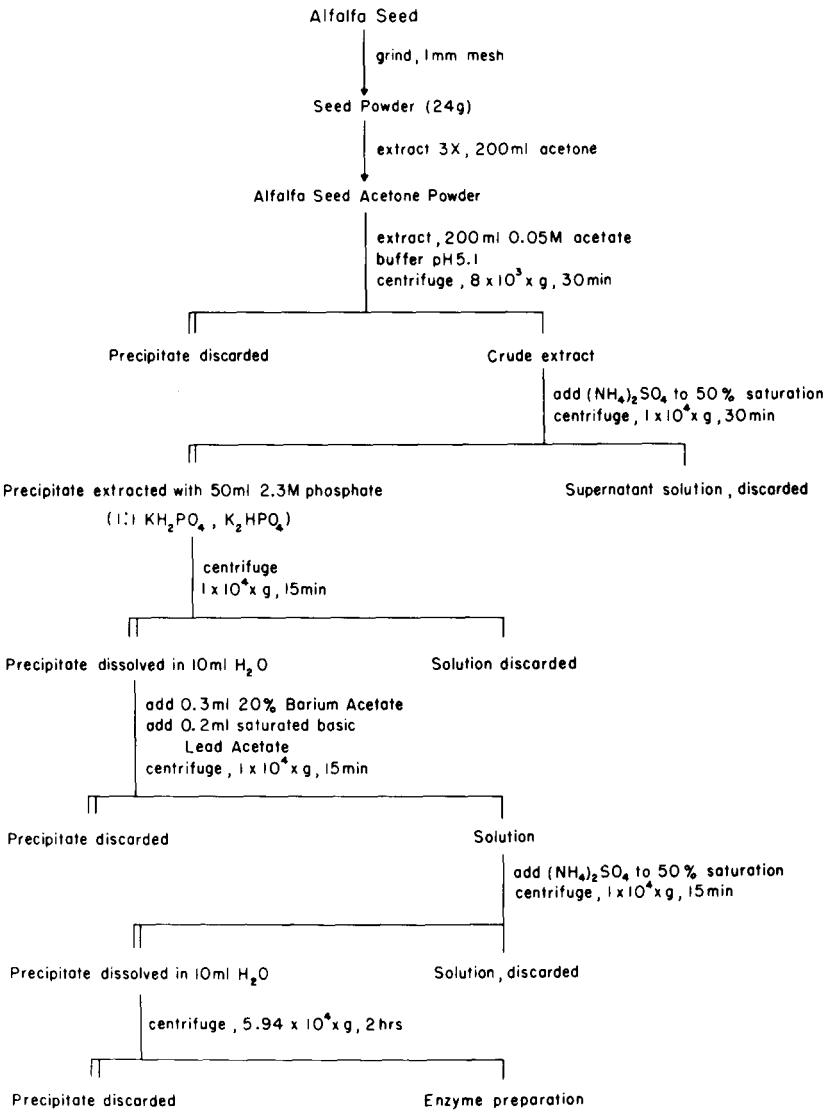


FIG. 1. Flow diagram of the isolation procedure for alfalfa seed lipoxygenase.

30% 9- and 70% 13-hydroperoxy linoleic acid. These findings are in agreement with the earlier values reported by Hamberg and Samuelsson (8).

Hydroperoxides produced by catalytic oxidation of linoleic acid using alfalfa lipoxygenase were analyzed with the same methods as used in the soybean lipoxygenase. The results are shown in Figure 3. The areas under 317 and 373 were 48% and 52% which are the distribution of 9- and 13-hydroperoxides, respectively. Results obtained from separate enzyme isolations 2 and 3 (Table II) were 49% 9- and 51% 13- and, 52% 9- and 48% 13-hydroperoxide, respectively.

DISCUSSION

Siddiqi and Tappel (5) reported no success in attempts to purify lipoxygenase from expressed alfalfa leaf sap. The methods used in this report were developed in an attempt to concentrate lipoxygenase from acetone powders of alfalfa seeds free of hydroperoxide-decomposing enzymes. All of the steps outlined in Figure 1 have been used in various combinations by earlier workers (25) in isolations from other enzyme sources.

The widely used heat treatment (4,11,12,25) to remove the hydroperoxide-decomposing enzymes was not used in this method since the

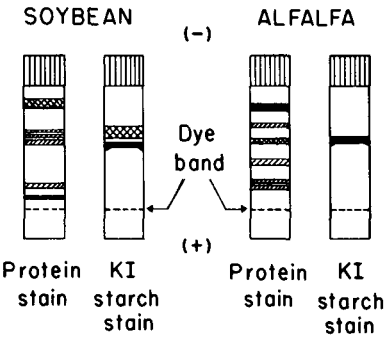


FIG. 2. Disc gel electrophoresis of 150 μg of soybean and 200 μg of alfalfa lipoxygenase. Protein was visualized with Coomassie Brilliant Blue and the active enzyme was visualized with a potassium iodide starch stain (see text).

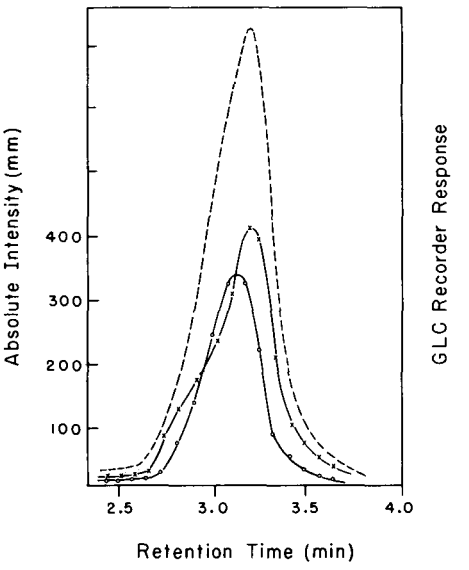


FIG. 3. Plot of mass spectral intensities vs GLC retention time of the reduced and derivatized products of alfalfa lipoxygenase. See text for methods used in reduction and derivatization. o, m/e 317 (9-OTMS-TMS-stearate); x, m/e 373 (13-OTMS-TMS-stearate); -, GLC peak.

application of heat invariably led to the concomitant destruction of alfalfa lipoxygenase at comparable rates. The combination of phosphate precipitation and heavy metal ion treatment led to the complete removal of the hydroperoxide-destroying enzymes with a recovery of 24% of the initial lipoxygenase activity. The assays at intermediate stages of purification showed evidence of interfering nonprotein substances which were eliminated by the second ammonium sulfate precipitation and the centrifugation of the final enzyme solution. No information is available on the nature of this interference.

Since several activity bands appeared on disc gel electrophoresis of lipoxygenases isolated from wheat and soybean, Guss et al. (21) suggested the existance of isoenzymes. In the stained gels of alfalfa lipoxygenase all of the activity was found in one band while the product was equally distributed between the 9- and 13-linoleate hydroperoxide isomers. Electrophoresis of a soybean preparation showed an activity distribution similar to that reported by Guss et al. The data obtained with enzymes isolated from alfalfa do not support the concept of separate isoenzymes being responsible for the production of the 9- and 13-isomers.

Recently, Zimmerman (11) reported that the percentage of 9-hydroperoxy compound varied from 6% to 30% and suggested that the 9-isomer occurs from autooxidation. Initially, experiments in our laboratory using one scan of the GLC peaks to determine the product distribution of alfalfa lipoxygenase led to the erroneous conclusion that only the 13-isomer was formed. However, using the technique outlined in this paper, we found that the ratio of the 13-isomer to the 9-isomer was about 50:50. These results were reproducible throughout three different enzyme isolations and product analyses. Our result showed that the 9- and 13-isomers vary somewhat in volatility (Fig. 3), thus making the spectrum dependent on the exact moment of mass spectral scanning. Multiple scanning of the GLC peak eliminates this problem. The 10- and 12-hydroxy isomers showed less evidence of separation on the GLC column. The reproducible results from three individual enzyme preparations, and the absence of 11-hydroperoxide isomers in our analysis as well as in reports by other workers, countermand the possibility of autooxidation. The reported variations in distribution between the 9- and 13-hydroperoxy-isomers may be explained on the basis of differences in volitility influencing the direct probe and GLC-MS measurements made by previous workers.

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Studies on the Hydrolysis and Utilization of Long Chain Acyl CoA Thioesters by Liver Microsomes

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ABSTRACT

Acyl CoA thioester "hydrolase" activity exhibited by liver microsomal preparations using a spectrophotometric assay for released CoA has been shown to be a composite of several enzymatic reactions. Pig liver microsomes form a considerable amount of triacylglycerol from endogenous precursors, with essentially all acyl groups being incorporated into the 1,3 positions. Rat and rabbit liver microsomes form very little triacylglycerol under the same conditions. The true acyl CoA hydrolase activity of pig liver microsomes is relatively insensitive to diisopropylfluorophosphate (DFP), whereas this compound strongly inhibits the rat and rabbit enzymes. The hydrolase remaining in the liver microsomes after DFP treatment is rapidly inactivated when incubated in the presence of substrate.

INTRODUCTION

Microsomal preparations contain most of the enzymes involved in glycerolipid metabolism and are often used with exogenous substrates to study various of these reactions. The acylation of lysophospholipids by such preparations has been studied rather extensively. Lands and Hart (1) described a continuous spectrophotometric method based on the release of free CoA from the acyl thioester which is often used to follow these reactions. This method has also been employed to study other acylation reactions involving CoA (2,3). One of the problems encountered in using this assay is the presence of an acyl CoA "hydrolase" activity in the microsomes. Lands and Hart found that the hydrolase in most microsomes could be reduced by diisopropylfluorophosphate (DFP) treatment, though this was not true in all cases. Our recent studies led us to examine the nature of this endogenous activity in more detail. In pig liver microsomes it has been found to be a combination of several reactions, the most prominent of which is formation of triacylglycerols (TG) by acylation of endogenous precursors, while that in rat liver microsomes is mostly true hydrolase (3.1.2.?:long chain acyl CoA hydrolase). This report describes some of the properties of the enzymes catalyzing these reactions.

MATERIALS AND METHODS

The microsomes used in these studies were prepared from livers of female Sprague-Dawley strain rats maintained on a standard Lab Chow diet and from livers of freshly-slaughtered pigs from a local abattoir. Tissues were homogenized in 0.25 M sucrose containing 0.001 M EDTA (9 ml/g of liver). The homogenates were centrifuged and the pellets sedimenting between 225,000 and 3,150,000 *g*-min (calculated for *g*-max) were resuspended in fresh sucrose-EDTA and immediately frozen at -20 C until used. Rat liver microsomes prepared in this way contained less than 4% of the total monoamine oxidase, about 15% of the acid phosphatase, 7% of the uricase, 72% of the glucose-6-phosphatase and 68% of the esterase activities in the total homogenates.

Rates of acyltransfer from acyl-CoA to an acceptor or of hydrolysis of the thioester were measured by the reaction of the sulfhydryl group in the liberated CoA with 5,5'-dithiobis-(2-nitrobenzoate) (DTNB) (1,4). The resultant increase in absorbance at 413 nm was continuously monitored in a Gilford Model 2000 recording spectrophotometer. Alternatively, ^{3}H-palmitoyl CoA was used as the substrate, and the reaction stopped at the appropriate time by addition of 5 volumes of chloroform-methanol, 2:1 (5). The extracted lipids were separated by thin layer chromatography (TLC), each fraction scraped from the plates and the radioactivity determined in a Nuclear-Chicago liquid scintillation spectrometer (6).

The standard assay mixture contained 30 to 35 nmoles of acyl CoA, 1 mg of enzyme protein and 1 mmole of DTNB in 1 ml of 0.1 M Tris-Cl, pH 7.4. All assays were performed at 30 C.

Acyl CoA thioesters were prepared as described by Reitz and Lands (7). 1-Acyl glycerylphosphorylcholine (acyl GPC) was prepared from egg lecithin by hydrolysis with snake venom (8).

TABLE I

Incorporation of the Acyl Group of ^{3}H-Palmitoyl CoA Into Lipid Fractions of Pig Liver Microsomes[a]

	5 Sec	1 Min	2 Min	4 Min	5 Min	7 Min	10 Min
CE[b]	0.07[c]	0.06	0.17	0.35	0.25	0.16	0.19
TG	0.24	2.66	6.08	8.36	9.25	11.54	9.74
FFA	0.25	0.80	1.74	2.44	3.32	3.70	4.46
DG	0.30	2.08	2.53	1.53	1.53	1.82	1.46
PL	0.20	0.52	0.93	0.94	0.53	0.73	0.67
Total	1.06	6.12	11.45	13.62	14.88	17.95	16.52
Total 16:0 CoA used		8.0	11.5	14.1	14.7	15.8	17.0

[a]Pig liver microsomes (1.04 mg of protein) were incubated with 33 nmoles of ^{3}H-palmitoyl CoA (1050 cpm/nmole) as described in the Methods section. At the indicated times, chloroform-methanol was added to stop the reaction, the lipids isolated, separated by TLC, and the radioactivity in each fraction determined. The total 16:0 CoA utilized was determined in two similar samples using the spectrophotometric assay.

[b]Abbreviations: CE, cholesterol esters; TG, triacylglycerols; FFA, free fatty acids; DG, diacylglycerol; PL, polar lipids.

[c]Nmoles incorporated.

RESULTS

While using the DTNB assay to study the acylation of lysophospholipids by microsomal preparations, we consistently observed a difference in the time course of acyl CoA utilization or hydrolase, by enzymes from rat and pig livers prior to addition of exogenous substrate. With both there was an initial rapid rate of release of free CoA on addition of the ester which declined after a few minutes of incubation. If additional acyl CoA was added to the rat liver preparation, the rate was restored to the initial velocity, while the activity continued to decline with the pig liver enzyme, reaching a much slower, steady rate (Fig. 1). This did not appear to be a generalized loss of activity, as these preparations maintained the ability to rapidly acylate acyl GPC over much longer time periods.

Incubation of the pig liver enzyme with ^{3}H-palmitoyl CoA for 10 min and isolation of the radioactive products demonstrated that much of the activity was associated with the TG fraction. A further study of the time course of appearance of label in the various lipid fractions showed that much of the early rate of appearance of free CoA in the Gilford assay could be attributed to formation of TG, while the later steady rate was apparently due to true hydrolase activity (Table I). While hydrolysis of newly-formed radioactive TG by the microsomal lipase described by Muller and Alaupovic (9) might contribute to the formation of the radioactive free fatty acids (FFA), comparison with data from the spectrophotometric assay (Table I) indicates that this could only account for a small portion, if any, of the ^{3}H-FFA formed during the assay period. Some diacyl-

TABLE II

Incorporation of the Acyl Group of ^{3}H-Palmitoyl CoA Into Lipid Fractions of Rat and Rabbit Liver Microsomes[a]

	5 Sec		2 Min		5 Min		10 Min	
	Rat[b]	Rabbit[c]	Rat	Rabbit	Rat	Rabbit	Rat	Rabbit
CE[d]	0.03[e]	0.05	0.14	0.04	0.15	0.05	0.12	0.13
TG	0.08	0.11	0.29	0.25	0.20	0.26	0.45	0.44
FFA	0.57	0.50	3.38	1.22	9.19	2.46	16.70	5.56
DG	0.23	0.38	0.28	0.37	0.37	0.71	0.45	1.29
PL	0.55	0.34	0.97	0.68	0.79	0.96	1.28	1.63
Total	1.46	1.38	5.06	2.56	10.70	4.44	19.00	9.05

[a]Same procedure as in Table I.

[b]Rat liver microsomes, 1.18 mg of protein.

[c]Rabbit liver microsomes, 0.99 mg of protein.

[d]Abbreviations: see Table I.

[e]Nmoles incorporated.

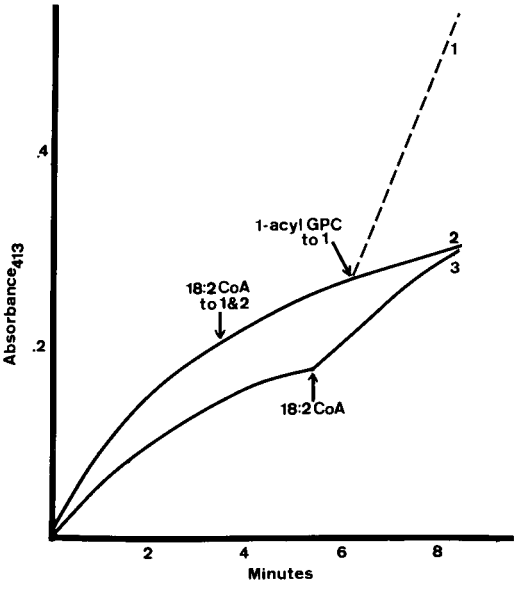

FIG. 1. Time course of metabolism of linoleoyl CoA by pig and rat liver microsomes. Pig (curves 1 and 2) or rat (curve 3) liver enzyme was incubated with linoleoyl CoA as described in the Methods section. Additional acyl CoA or 150 nmoles of 1-acyl GPC was added where indicated.

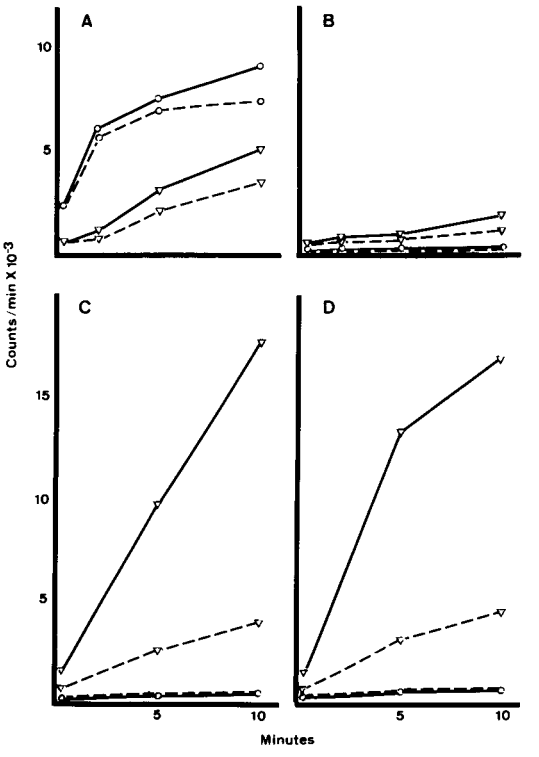

FIG. 2. Effect of DFP treatment on the kinetics of labeling of triacylglycerols and free fatty acids from ^{3}H-palmitoyl CoA by pig and rat microsomes. A, pig enzyme pretreated at 0 C for 60 min before assay. C, rat enzyme treated in same manner. B, pig enzyme pretreated at 22 C for 60 min before assay. D, rat enzyme treated in same manner. ○, triacylglycerols; ▽, free fatty acids; Solid lines, control; dashed lines, pretreated with 1 mM DFP. See Table I for incubation and assay conditions.

glycerols (DG), polar lipids (PL) and a very small amount of cholesterol esters were also formed, but after 1 min their contribution to the total activity was negligible. In similar experiments with rat and rabbit liver microsomes, most of the activity was recovered in FFA (Table II).

The labeling data obtained with the pig liver microsomal preparation suggested that the TG produced probably result from acylation of preexisting DG or DG produced by acylation of MG, rather than from DG newly-formed via phosphatidic acid. Pancreatic lipase hydrolysis (10) of a sample of these TG isolated by Florisil chromatography (11) indicated that essentially all of the labeled fatty acid incorporated was in the 1,3 positions. Pig liver microsomes (7.9 mg of protein) were incubated with 200 nmoles of ^{3}H-palmitoyl CoA (210,000 counts/min) for 10 min at 30 C. The TG (117,800 counts/min) were purified from the extracted lipids by chromatography on Florisil and an aliquot submitted to pancreatic lipase hydrolysis for 10 min. Products of this hydrolysis were separated by TLC and the radioactivity in each fraction determined. The distribution of radioactivity in products of lipase hydrolysis of triacylglycerols synthesized by pig liver microsomes was the following: Unhydrolyzed TG, 2601 counts/min; FFA, 9034; DG, 427; and MG, 43.

DFP treatment has been reported to remove most of the hydrolase activity from rat liver microsomes (1). The procedure described by Lands and Hart calls for incubation of the microsomes with 1 mM DFP at room temperature for 1 hr. Such treatment indeed caused a drastic reduction in hydrolase activity with the rat liver preparation, with essentially no change in the already very small incorporation of ^{3}H 16:0 into TG (Fig. 2D). However, with the pig enzyme both hydrolase activity and TG synthesis were much lower in both controls and DFP-treated preparations as compared to these activities in freshly-thawed microsomes (Fig. 2B). When the same experiment was run with the DFP treatment performed in ice, results with the rat liver preparation were similar to those at 22 C (Fig. 2C). With the pig liver enzyme, there was little loss in TG synthesis, while the production of FFA in the controls was essentially the same as in freshly-thawed

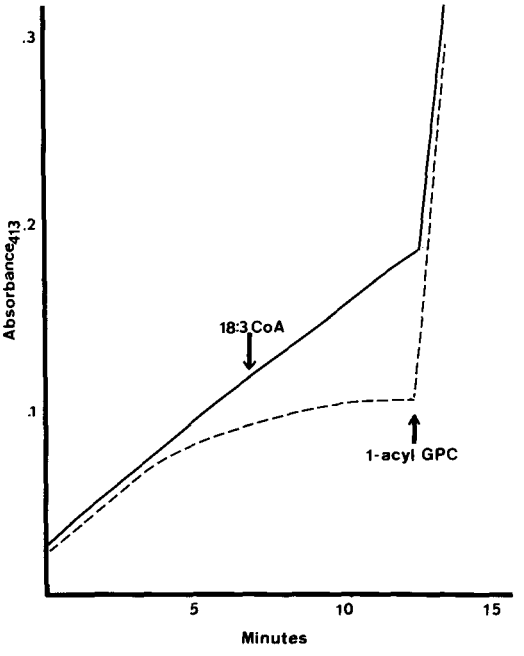

FIG. 3. Effect of DFP treatment on acyl CoA metabolism by rat liver microsomes. Rat liver enzyme was preincubated at 22 C for 60 min alone and in the presence of 1 mM DFP, then assayed in the standard spectrophotometric assay with linolenoyl CoA. Additional acyl CoA or 150 nmoles of 1-acyl GPC was added to both preparations where indicated. Solid line, no DFP; dashed line, 1 mM DFP.

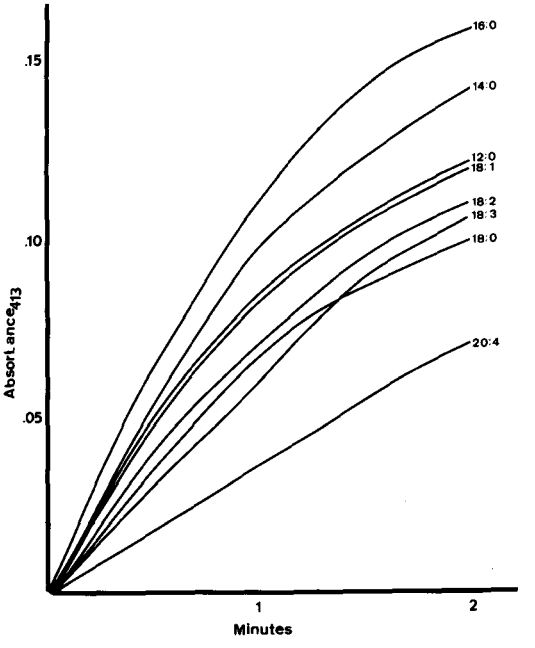

FIG. 4. Initial kinetics of production of free CoA from different acyl CoA derivatives by pig liver microsomes. The standard spectrophotometric assay was employed. The acyl CoA derivatives were present in initial concentrations of 27 to 30 μM.

microsomes and was reduced only slightly by DFP (Fig. 2A).

The kinetic behavior of the rat liver microsomal hydrolase remaining after DPF treatment was also altered, rather than the effect being a simple reduction in the rate (Fig. 3). After treatment, hydrolysis ceased after a few minutes of incubation and was not restored by addition of more acyl CoA, as was true with the untreated enzyme. On the other hand, the ability of these preparations to acylate acyl GPC was not impaired by the DFP treatment.

When the pig liver enzyme was exposed to different acyl CoA esters at the same substrate concentrations normally used for the acyl CoA-acyl GPC acyltransferase assay, the reaction progress curves were quite different, with palmitoyl CoA being the best substrate and arachidonoyl CoA the poorest (Fig. 4).

The effect of substrate concentration on the initial reaction rates was then determined for several acyl CoA esters. These assays were performed in the presence of sonicated microsomal lipid, which, as Abou-Issa and Cleland (12) have shown, forms mixed micelles with acyl CoA to prevent substrate inhibition of rat liver acyl CoA L-glycerol-3-phosphate acyltransferase. It served a similar role here, at least with the saturated derivatives, which produced typical substrate saturation curves under these conditions but exhibited substrate inhibition in the absence of the lipid. Palmitoyl CoA yielded the greatest maximum velocity and stearoyl CoA the lowest (Fig. 5A). The Vmax for palmitoyl CoA determined from a Michaelis plot was approximately 20 μmoles/min with an apparent Km of about 9 μM. On the other hand, unsaturated acyl CoA esters all produced curves indicating substrate inhibition at rather low concentrations (Fig. 5B).

DISCUSSION

The hydrolase activity which is seen in the spectrophotometric assay for acyltransferases described by Lands and Hart (1) is not due to a single enzymatic activity. This is especially true with fresh pig liver microsomal preparations, where a considerable amount of TG is formed from endogenous precursors. When these preparations are preincubated at room temperature for an hour, very little TG is formed. This may be a result of acylation of the endogenous precursors with fatty acids already present in the microsomal preparation. It may also be partially due to loss of enzymatic activity, as

the hydrolase activity also decreases. However, acylation of 1 acyl GPC does not decrease during this time. Marinetti (13) has reported that enzymes involved in TG and DG synthesis are more unstable than those for phospholipid synthesis in rat liver homogenates treated with puromycin. This may also be true with the pig liver microsomes.

DFP treatment of rat liver microsomes not only reduced the hydrolase activity, as reported by Lands and Hart (1), but also caused a complete loss of activity after several minutes of incubation with substrate. It is possible that the DFP does not completely block the hydrolase activity, but alters the configuration of the enzyme sufficiently to produce an increased susceptibility to denaturation by the detergent action of the acyl CoA. However this inactivation occurs, it makes possible the study of acyltransferases with lysophospholipid substrates in the absence of any contaminating activities, utilizing a combination of DFP treatment and preincubation of the enzyme with acyl CoA. This is also true with the pig liver enzyme, though in this case the true hydrolase activity is minimal and preincubation with acyl CoA to remove endogenous substrates alone yields a very low consistent hydrolase activity, which can be easily corrected.

The TG which are synthesized by the pig liver microsomal preparations have essentially all of the incorporated fatty acid in the 1 and 3 positions. Akesson (14) has recently studied the synthesis of TG from exogenous DG dispersed in nonionic detergent by pig liver microsomes using an acyl CoA generating system and labeled fatty acids. In this system he also observed an incorporation of fatty acid into TG in the absence of added DG of the same order found in our studies. Exogenous 1,2- and 2,3-DG were equally good substrates for the acylation, equal amounts of label appearing in the 1 and 3 positions with a *rac*-1,2-DG substrate. His data indicated the presence of enzymes in these microsomes which can hydrolyze the ester bond at the 1 position of DG, and presumably of TG. Muller and Alaupovic (9) have demonstrated the presence of a microsomal triacylglycerol lipase, but did not study its positional specificity. The endogenous DG in the pig liver microsomes could, therefore, be a mixture of the 1,2- and 2,3-isomers. The label appearing in the DG is probably due to acylation of 2-acylglycerols produced by these lipases. Sundler and Akesson (15) have found that pig liver microsomes specifically acylate exogenous 2-acylglycerol at position 1. *Rac*-1-acylglycerol is also acylated but at only a small fraction of the rate with the 2-isomer.

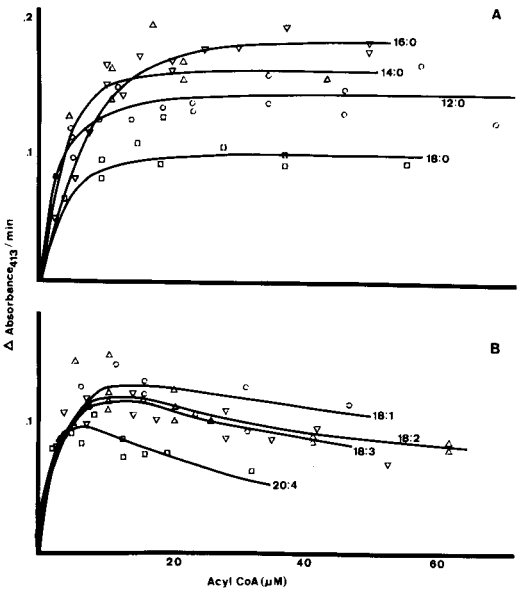

FIG. 5. Initial velocity of free CoA production by pig liver microsomes as a function of acyl CoA concentration. A, saturated acyl CoA derivatives; ○, 12:0; △, 14:0; △, 16:0; □, 18:0. B, unsaturated acyl CoA derivatives: ○, 18:1; △, 18:2; ▽, 18:3; □, 20:4. All measurements were made using the standard spectrophotometric assay in the presence of sonicated total pig liver microsomal lipid (20 μg Pi/cuvette). Each point represents a single determination.

The relative specificities observed for different acyl CoA esters during the initial portion of the spectrophotometric assay obviously result from a combination of the properties of more than one enzyme, as the amount of DG formed in the first minute is almost as great as that of TG (Table I). That relatively smooth substrate saturation curves were obtained for such a complex system is not particularly surprising, as we have obtained similar results for the incorporation of glycerol into total lipids by rat liver slices (16). The substrate inhibition seen with the unsaturated acyl CoA esters, even in the presence of sonicated phospholipid, may reflect a preferential action on one of the enzymes present. Experiments to determine the resultant distribution of labeled acyl groups transferred from unsaturated acyl CoA esters at various substrate levels will be necessary to determine what is actually occurring under these experimental conditions.

The presence of endogenous acceptors for TG synthesis has also been noted in microsomes from chicken adipose tissue (17) and chicken liver (18). However, we failed to find any significant amounts of such acceptors in rat or rabbit liver microsomes. It is interesting that both of these preparations also have high, DFP

sensitive acyl CoA hydrolase activities. The significance of these latter findings is not presently understood, but they serve as a reminder that species or class differences must be taken into account in any metabolic study.

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The Inhibitive Effects of Azasterols on Sterol Metabolism and Growth and Development in Insects With Special Reference to the Tobacco Hornworm[1]

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ABSTRACT

Several monoazasterols were found to be potent inhibitors of Δ^{24}- and $\Delta^{22,24}$-sterol reductase enzyme(s) in the tobacco hornworm, *Manduca sexta* (Johannson). Certain of these inhibitors also prevented normal larval development and pupation in the hornworm at dietary concentrations in the parts per million range. Comparative studies with several different insects indicated differences between the species with respect to the effects of the azasterols. The relationship of azasterol structure to the inhibitory effect(s) on sterol metabolism and larval development is discussed.

INTRODUCTION

Since the discovery that desmosterol (24-dehydrocholesterol) is an intermediate in the conversion of β-sitosterol to cholesterol in the tobacco hornworm, *Manduca sexta* (Johannson) (1) several vertebrate hypocholesterolemic agents, including the two diazasterols, 22,25- and 20,25-diazacholesterol (Fig. 1 I,II), have proved most useful in studying the utilization and metabolism of phytosterols in insects. Two inhibitors of cholesterol biosynthesis, 22,25-diazacholesterol and triparanol (MER-29), were used to first demonstrate that the conversion of β-sitosterol to cholesterol can be inhibited and growth and development disrupted in the tobacco hornworm (2). This block occurs primarily through the inhibition of a Δ^{24}-sterol reductase enzyme(s) and results in an accumulation of desmosterol and a reduction of cholesterol formation.

We have further demonstrated that desmosterol is a common intermediate in the conversion of a number of different phytosterols to cholesterol in the hornworm by using 20,25-diazacholesterol to block the Δ^{24}-sterol reductase system (3). The usefulness of azasterols again became apparent in our studies when a new sterol, 22-*trans*-cholesta-5,22,24-trien-3β-ol, was isolated and identified as an intermediate in the conversion of stigmasterol to cholesterol in several insects (4). This sterol was found to accumulate when a diazasterol was fed in combination with stigmasterol. In addition, the identification of fucosterol as a metabolite of β-sitosterol and a probable intermediate in its conversion to cholesterol in the hornworm was also aided by the use of 20,25-diazacholesterol (5).

When certain azasterols are administered in the diet, hornworm larvae experience difficulty in molting, some form prepupae an instar early and many insects are unable to pupate. Comparative studies with diazasterols and other inhibitors of Δ^{24}-sterol reductase enzymes, such as triparanol and 3β-hydroxy-Δ^{5}-norcholenic acid (3β-hydroxy-24-norchol-5-en-23-oic acid) (6), have indicated that the disruption of molting and development caused by the azasterols is brought about by effects other than solely a limitation in cholesterol formation. Over 20 azasterols have been tested for their effects on sterol metabolism and growth and development in the tobacco hornworm, and these studies have been com-

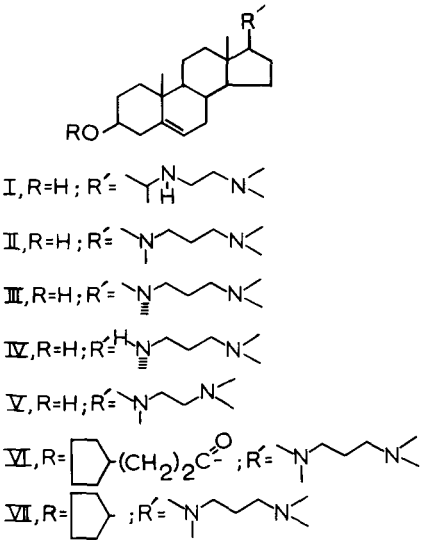

FIG. 1. Azasterol structures.

[1]One of 12 papers to be published from the "Sterol Symposium" presented at the AOCS Meeting, New Orleans, April 1970.

TABLE I

Sterol Composition of Tobacco Hornworms Fed Various Concentrations of 20,25-Diazacholesterol in Combination With β-Sitosterol

Inhibitor concentration[a] (% wet wt.)	Per cent of total sterols[b]		Dealkylated sterols
	Cholesterol	Desmosterol	
Control	84.5	<2.0	86.5
0.00165	40.0	44.2	84.2
0.0033	26.8	41.4	68.2
0.0065	16.3	51.7	68.0
0.013	13.2	42.0	55.2
0.026	5.6	21.8	27.4

[a]All diets contained β-sitosterol at 0.026% wet weight.

[b]Remainder of sterol was unchanged dietary sterol.

plemented with similar tests on other insects which convert C_{28} and C_{29} plant sterols to cholesterol. From these studies a considerable insight into the relationship of structure to the activity of azasterols in insects has been obtained. This paper describes the results of these studies and also reports the species differences found with respect to the effects of the azasterols on sterol metabolism as well as on molting and development.

EXPERIMENTAL PROCEDURES

The bioassay used to test azasterols for inhibitory activity in the hornworm was the same as previously described (2). β-Sitosterol, the sole added dietary sterol, unless otherwise noted, was coated on the dry components to achieve a concentration of 0.026% wet weight (0.2% dry weight) in the hornworm larval diet. All dietary sterols were >99% pure by gas liquid chromatography (GLC) and thin layer chromatography (TLC) analyses. Larvae of the corn earworm, *Heliothis zea* (Boddie) and fall armyworm, *Spodoptera frugiperda* (J.E. Smith) were reared on the same diet. The sterol was coated on diets (7) for the German cockroach, *Blattella germanica* (L.) and American cockroach, *Periplaneta americana*(L.) at a concentration of 0.2% dry weight. A modification of the cockroach diet was also used with the firebrat, *Thermobia domestica* (Packard). Initial tests with each azasterol were made with the compound at the same concentration as β-sitosterol. To accurately assess the inhibitive effects of certain of the more potent azasterols, subsequent tests with lower or higher concentrations were carried out as indicated by preliminary results. The azasterols were kindly provided by G.D. Searle and Co., Chicago, except for N-methyl-N-(3-dimethylamino)-propyl-17α-amino-androst-5-en-3β-ol (III) and compound IV which were furnished by R.E. Counsell, University of Michigan, Ann Arbor. All azasterols except compounds III, IV, VI and VII were tested as the hydrochloride salts.

In addition to the effects on growth and development, the sterols of insects reared on the test diets were examined to determine the effects of the azasterol on sterol metabolism. Hornworms, corn earworms and fall armyworms were frozen as prepupae or as retarded larvae. German and American cockroaches and firebrats were also frozen either as retarded nymphs or adults at appropriate periods of time. The sterols were isolated and analyzed by column chromatography, TLC and GLC as previously described (1). Unisil (100-200 mesh, Clarkson Chemical Co., Williamsport, Pa.) and Woelm neutral grade II alumina (Alumina Woelm, Alupharm Chemicals, New Orleans) were the adsorbents used for column chromatographic separations. Quantitation of sterols was carried out on a Barber-Colman Model 10 gas chromatograph using 0.75% SE-30 coated on Gas-Chrom P.

RESULTS

Data in Table I show a relationship between the dietary concentrations of 20,25-diazacholesterol and the composition of the sterols isolated from the hornworm. An examination of the relative quantity of the total dealkylated sterols (i.e., cholesterol plus desmosterol) indicates an overall decrease in dealkylation at the higher concentrations of the inhibitor probably due either to a feedback effect or an additional block in the pathway. This azasterol effects larval growth and development only at concentrations of 0.013% (wet weight) or greater. The 22,25-diazacholesterol was found to produce effects nearly identical to those caused by the 20,25-diazacholesterol. The

TABLE II

Effect of Various Azasterols Fed in Combination With β-sitosterol on the Sterol Composition and Larval Development of Tobacco Hornworms

Compound[a]	Per cent of total sterols[b]		Inhibition of development[c]
	Cholesterol	Desmosterol	
II	5.6	21.8	++
III	7.9	51.1	+
IV	18.2	56.2	0
V	5.5	26.0	++++
VI	1.4	44.5	++++
VII	3.0	27.2	++++

[a]All diets contained the azasterol and β-sitosterol each at 0.026% wet weight.

[b]Remainder of sterol was unchanged dietary sterol.

[c]Six insects per test, tests replicated two to six times. Inhibition rated as follows: 0 = no effect on development; + = less than one half of the insects develop to normal prepupae, larval growth retarded; ++ = less than one third of the insects develop to normal prepupae, some abnormal fourth instar prepupae formed; +++ = less than one sixth of the insects develop to normal prepupae, approximately one third of the insects form abnormal fourth instar prepupae; ++++ = no development beyond abnormal fourth instar prepupae, considerable mortality during the first three larval molts.

saturated analog of 22,25-diazacholesterol, however, had a less inhibitory effect on larval development even though it brought about a change in sterol composition similar to that found with the Δ^5-analog. Several 20,25-diazasterols with supernumerary carbons at the 26 and 27 positions, or which included the nitrogen at the 25 position in a ring structure were also tested in the hornworm; some of these compounds inhibited the desmosterol reductase system and severely reduced the amount of cholesterol available, but had no appreciable effect on larval development.

The 17α-epimer of 20,25-diazacholesterol (III) was somewhat less potent than the 17β-epimer (II) as an inhibitor of development, and was also less effective in blocking dealkylation (Table II). The 17α-analog lacking the 21-methyl (IV) was a less effective inhibitor of sterol metabolism and had essentially no effect on development. Interestingly, 20,24-diaza-25-norcholesterol (V) with one less carbon in the side chain inhibited cholesterol formation and blocked dealkylation to about the same extent as did the 20,25-diazacholesterol, but, based on tests at lower concentrations, it was about four times more active in blocking larval development. The cyclopentyl propionic acid ester (VI) of the 20,25-diazacholesterol was approximately three times as inhibitory to larval development as the parent compound, but the amount of dealkylated sterol was greater with VI, as evidenced by the large accumulation of desmosterol. A cyclopentyl ether derivative (VII) was over five times as inhibitory to larval development as the parent diazacholesterol. Compounds II, V, VI and VII were all inhibitory to the German cockroach when fed in combination with β-sitosterol, and as in the hornworm, of these four azasterols, compound VII was the most potent inhibitor of development (Table III). The relative amounts of cholesterol found in treated cockroaches were much higher than those found in hornworms even though nymphal development was severely disrupted in the cockroaches.

A series of monoazacholesterols was tested

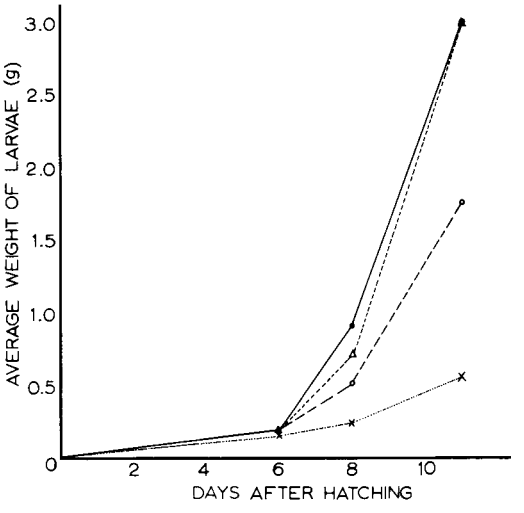

FIG. 2. Growth rates of tobacco hornworm larvae reared on diet containing β-sitosterol alone (control) ●——●, or in combination with 8 ppm of either 23-azasterol ○- - -○, 24-azasterol Δ- - -Δ, or 25-azasterol X. . . .X. All diets contained β-sitosterol at 0.026% wet weight.

TABLE III

Effect of Various Azasterols Fed in Combination With β-Sitosterol on the Sterol Composition and Nymphal Development of German Cockroaches

Compound[a]	Per cent of total sterols[b]		Inhibition of development[c]
	Cholesterol	Desmosterol	
Control	92.8	<1.0	
II	47.3	33.2	+
V	57.9	27.9	+
VI	57.2	25.7	+
VII	79.6	9.7	++
23-Azacholesterol	47.0	17.9	++
24-Azacholesterol	42.5	8.5	++
25-Azacholesterol	47.1	8.6	++

[a]All diets contained the azasterol and β-sitosterol each at 0.2% dry weight except compound VII which was tested at 0.05%.

[b]Remainder of sterol was unchanged dietary sterol.

[c]Twenty insects per test. Inhibition ratings based on nymphal weights as compared with controls, at end of 30 day test period: + = 30-80% weight reduction; ++ = >80% reduction in weight.

in the tobacco hornworm and as seen in Table IV, the extent of inhibition of sterol metabolism is dependent on the position of the nitrogen in the side chain. There is less total dealkylated sterol in the insects after feeding the 23-,24-, and 25-monoazasterols than with the diazasterols. Based on tests at lower concentrations, all three of these compounds are more potent inhibitors of larval development than any of the previously discussed compounds, and the 25-azacholesterol is the most active of the azasterols tested (Fig. 2). This compound at a concentration of 8 ppm in the diet severely disrupts larval development, no pupation occurs at 4 ppm, and a considerable decrease in the number of insects pupating occurs even at 2 ppm. The 25-azacholesterol at 16 ppm brought about formation of fourth instar prepupae in about half of the hornworm larvae. Larvae reared individually in 1 oz plastic cups, at the latter concentration, were examined daily and molting records were kept to definitely ascertain that fourth instar prepupae were formed.

The 23-,24- and 25-monoazasterols also inhibited the development of German cockroach nymphs (Table III). As observed with the hornworm, the 25-azacholesterol was also the most potent in the German cockroach. In addition, the relative percentage of cholesterol in these cockroaches is quite high as was found with compounds V, VI and VII.

The sterol composition of hornworms fed stigmasterol in combination with the monoazasterols emphasizes the importance of the position of the nitrogen in the side chain with respect to inhibition of either the Δ^{24}- or the $\Delta^{22,24}$-sterol reductase activity. From Table V it is apparent that 22-*trans*-cholesta-5,22,24-trien-3β-ol accumulates to the greatest extent with the 20- and 22-monoazasterols.

The effects of 20,25-diazacholesterol have been examined in several species of insects that convert β-sitosterol to cholesterol. Table VI summarizes some of these results, including the inhibitory effects on sterol metabolism and development observed when β-sitosterol and the diazacholesterol are fed in combination, each at 0.2% dry weight. Of the immature insects tested, the primitive firebrat nymph was least affected; little desmosterol accumulated and no effect on development was detected over a five month period. The development of German cockroach nymphs is slightly less inhibited than American cockroach nymphs at this concentration, and sterol metabolism in both species is more severely affected than in the firebrat. The American cockroach adults were placed on the test diet after molting from the last nymphal instar, the nymphs having been reared in the stock colony on a diet of dog food. These adults were held nearly seven months on the test diets, with the females producing oothacae during this period. It is apparent from Table VI that the cholesterol pool of the female has been considerably depleted because of the requirement for this sterol for ootheca production. Sterol turnover also occurs in the males, and some desmosterol has accumulated because of the reduction in conversion of β-sitosterol to cholesterol brought about by the diazasterol. All three of the phytophagous lepidopterous insects were severely affected by the diaza-

TABLE IV

Effect of Monoazasterols Fed in Combination With β-Sitosterol on the Sterol Composition and Larval Development of Tobacco Hornworms

Compound[a]	Per cent of total sterols[b]		Inhibition of development[c]
	Cholesterol	Desmosterol	
Control	84.5	1.5	
20-Azacholesterol	85.6	1.5	+
22-Azacholesterol	40.8	8.7	+
23-Azacholesterol	1.6	6.6	+++
24-Azacholesterol	1.7	5.7	++++
25-Azacholesterol	2.3	6.7	++++

[a]All diets contained the azasterol and β-sitosterol each at 0.026% wet weight.
[b]Remainder of sterol was unchanged dietary sterol.
[c]Inhibition ratings as in Table II.

cholesterol with respect to the dealkylation of β-sitosterol and its conversion to cholesterol as well as disruption of the larval molting cycle.

DISCUSSION

A number of conclusions can be drawn concerning the relationship of structure to the activity of azasterols with respect to their effects on sterol metabolism and growth and development in the hornworm. There are actually three interrelated effects brought about by these azasterol inhibitors: first, a severe inhibition of the Δ^{24}-sterol reductase system, which limits the amount of cholesterol available to the insect and is a necessary prerequisite for the effects on hornworm larval growth and development; second, a drastic reduction in dealkylation occurs with any azasterol that is a potent inhibitor of the hornworm molting cycle so that the major sterol present in the insect is unchanged dietary sterol; third, a disruption of normal molting and development may occur in this insect when there is a severe limitation in cholesterol formation. However, previous work with several inhibitors which do not interfere with development has shown that inhibiting cholesterol formation is not in itself sufficient to disrupt the molting cycle of the hornworm.

Our results point to a number of structural features that may affect the inhibitory activity of these azasterols. The monoazasterols are the most inhibitory of the compounds we have tested and the 25-azasterol is the most active of these. The tertiary nitrogen at the 25 position thus is more inhibitory than a secondary nitrogen at either the 23 or 24 position. The 25-azacholesterol is over 30 times more potent than either the 20,25- or 22,25-diazacholesterol in blocking hornworm development, indicating that the additional nitrogen apparently is responsible for some steric effect that decreases the inhibitory action(s) of these diazasterols. This relation of structure to activity in the hornworm generally parallels the results from studies with rats on the relative effectiveness of monoazasterols and diazasterols as hypocholesterolemic agents (8).

The addition of substituents larger than

TABLE V

Effect of Monoazasterols Fed in Combination With Stigmasterol on the Sterol Composition of Tobacco Hornworms

Compound[a]	Per cent of total sterols[b]		
	Cholesterol	Desmosterol	$\Delta^{5,22,24}$-Cholestatrienol
Control	82.6	<2.0	<1.0
20-Azacholesterol	41.0	<2.0	31.8
22-Azacholesterol	11.8	<2.0	35.0
23-Azacholesterol	8.0	15.6	10.1
24-Azacholesterol	6.9	8.5	6.3
25-Azacholesterol	6.2	11.4	3.1

[a]All diets contained the azasterol and stigmasterol each at 0.026% wet weight.
[b]Remainder of sterol was unchanged dietary sterol.

TABLE VI

Effect of 20,25-Diazacholesterol Fed in Combination With β-Sitosterol on the Sterol Composition and Development of a Number of Insects

Insect[a]	Per cent of total sterols[b]		Inhibition of development[c]
	Cholesterol	Desmosterol	
Firebrat nymphs	69.8	11.4	0
German cockroach nymphs	47.3	33.2	+
American cockroach nymphs	30.9	16.5	+
Female American cockroach adults	49.7	6.1	
Male American cockroach adults	83.0	4.0	
Fall armyworm larvae	2.8	34.9	+++
Tobacco hornworm larvae	5.6	21.8	++
Corn earworm larvae	1.0	18.6	++

[a]All diets contained the azasterol and β-sitosterol each at 0.2% dry weight.

[b]Remainder of sterol was unchanged dietary sterol. Controls of all species fed β-sitosterol alone contained 83% to 90% cholesterol and <2% desmosterol.

[c]Inhibition ratings are as follows: fall armyworm (10 insects per test), tobacco hornworm (6 insects per test), and corn earworm (10 insects per test) same as in Table II; German and American cockroach nymphs same as in Table III except that in the American cockroach tests 35 insects were used per test and the nymphs were weighed at the end of 80 day test period.

methyl groups at the 26 and 27 positions of the side chain renders the azasterol considerably less active, particularly with respect to inhibition of growth and development. A number of such compounds tested in the hornworm quite effectively blocked the Δ^{24}-reductase system, severely reduced cholesterol formation, but had little or no effect on larval growth. However, two other azasterols with side chains that also differ considerably from cholesterol were found to be active. Both 20,24-diaza-25-norcholesterol (with the shortened side chain) and the 17α-epimer of 20,25-diazacholesterol (III) were inhibitory. The 21-nor derivative (IV) of the 17α-epimer was considerably less effective, indicating that the 21-methyl group is essential for maximum activity. Neither a Δ^5-bond nor a free 3β-hydroxyl group appears to be essential for azasterol activity; the saturated analog of the 22,25-diazacholesterol is approximately as active as the Δ^5-compound and both an ether and an ester derivative of the 20,25-diazacholesterol were several times more active than the parent compound. It will be of interest to determine if comparable increases in activity will be found with the corresponding esters and ethers of certain of the active monoazasterols, such as 25-azasterol.

The differences observed in the effects of a diazasterol on several species of insects (Table VI) again warns against making broad generalizations on sterol metabolism and utilization in insects. Of the test insects, the firebrat was the least affected by this inhibitor, both with respect to the formation of cholesterol and the disruption of development. Even though the effects on the development of the cockroach nymphs were similar to those found with hornworms, the relative percentage of cholesterol found in these inhibited cockroaches far exceeds that found in the inhibited hornworms. Either the hornworm is better able to function and grow on a limited supply of cholesterol than cockroaches or else other inhibitory mechanisms are involved. Both cockroaches also differ significantly in that they do not show the overall inhibition of dealkylation found in the hornworm. Thus there is also a considerable difference between different insects in the specificity of the enzyme system(s) involved in the dealkylation and conversion of phytosterols to cholesterol. As previously mentioned (3), these differences in sterol metabolism in insects may well be due to adaptations to the sterols normally available in the natural diet of the insect.

Continuing research with the azasterol analogs and their derivatives will provide additional information on the structure-activity relationships of these inhibitors and should also prove useful in further elucidating the pathway(s) of phytosterol metabolism and utilization in insects. The azasterols are also apparently directly or indirectly involved in metabolic pathways other than those having to do with formation of cholesterol from phytosterols. It would appear that a part, if not all, of the sequence of conversion of phytosterols to cholesterol in the hornworm occurs in the tissues of the gut, since in vitro studies have shown that reduction of

desmosterol, the final step in this pathway occurs almost exclusively in the midgut tissues. However, in order for an azasterol to disrupt other pathways of steroid metabolism, such as ecdysone formation, it may be necessary for the azasterol to be transported to tissues other than the gut. Preliminary studies with radiolabeled azasterols indicate that extremely small quantities of these inhibitors are incorporated into other hornworm tissues, and perhaps valuable information concerning the various sites of steroid metabolism may be obtained from analyses of these tissues. Through the use of these radiolabeled azasterols we hope eventually to gain a better understanding of the action of these compounds—whether these inhibitors block other important metabolic pathways for steroids in insects such as ecdysone biosynthesis, or whether the azasterols are metabolized to other, biologically active compounds, or both.

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The Distribution of Sterols in Algae[1,2]

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ABSTRACT

Available analytical techniques are now sufficient for the separation and identification of sterols from complex mixtures in plants. Gas and thin layer chromatography and mass spectroscopy in particular, have been used to resolve some of the confusion concerning the sterol composition of algae. Red algae (Rhodophyta) contain primarily cholesterol, although several species contain large amounts of desmosterol, and one species contains primarily 22-dehydrocholesterol. Only a few Rhodophyta contain traces of C-28 and C-29 sterols. Fucosterol is the dominant sterol of brown algae (Phaeophyta), apparently the major sterol of every species examined. Most Phaeophyta also contain traces of cholesterol and biosynthetic precursors of fucosterol. The sterols of green algae (Chlorophyta) are much more varied and complex than those of other groups of algae. Whereas the Phaeophyta and Rhodophyta contain one primary sterol, many of the Chlorophyta contain a complex mixture of sterols such as occurs in higher plants. The Chlorophyta contain such sterols as chondrillasterol, poriferasterol, 28-isofucosterol, ergosterol, cholesterol and others. Sterol composition may be of value in the systematics of plants such as the Chlorophyta. Recently (for the first time) complex mixtures of sterols have been isolated in very small amounts in the blue-green algae (Cyanophyta). Available data on the sterols of other groups of algae are insufficient for making useful comparisons.

[1]One of 12 papers to be published from the "Sterol Symposium" presented at the AOCS Meeting, New Orleans, April 1970.

[2]Scientific Article No. A2606, Contribution No. 4331 of the Maryland Agricultural Experiment Station.

INTRODUCTION

Since their recent isolation from bacteria (1,2) and blue-green algae (3,4), sterols have

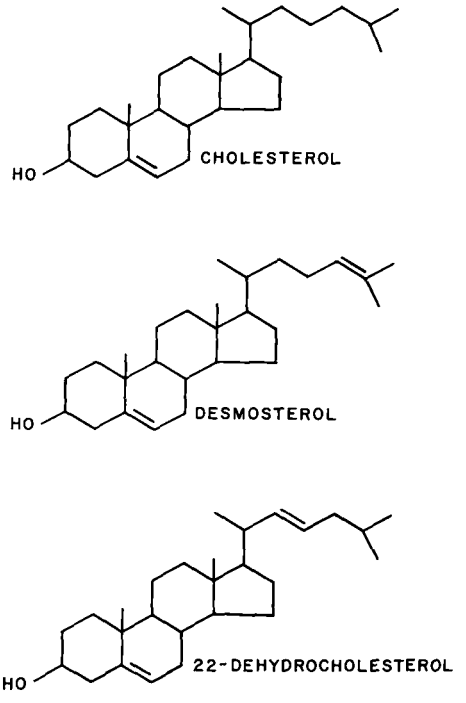

FIG. 1. Sterols of red algae.

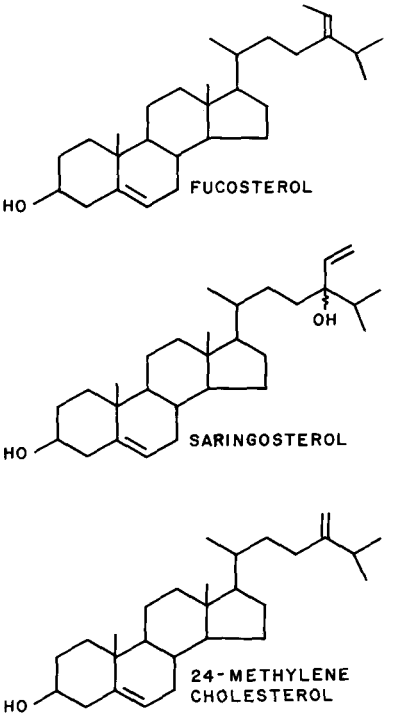

FIG. 2. Sterols of brown algae.

TABLE I

Sterols of Red Algae (Rhodophyta)

Order	Species	Major sterols[a]	References
Porphyridiales	*Porphridium cruentum*	none	12
Bangiales	*Porphyra purpurea*	desmo	13
Gelidiales	*Acanthopeltis japonica*	chol	32
	Gelidium amansii	chol	32
	G. japonicum	chol	33
	G. subcostatum	chol	33
	Pterocladia tenuis	chol	33
Cryptonemiales	*Dilsea earnosa*	chol	13
	Corallina officinalis	chol	13
	Gloiopeltis fureata	chol	34
	Tichocarpus crinitus	chol	34
	Grateloupia elliptica	chol	34
	Cyrtymenia sparsa	chol	34
	Polyides caprinus	chol	13
	P. rotundus	chol	11
Gigartinales	*Gracilaria verrucosa*	chol	b
	Plocamium vulgare	chol	13
	Furcellaria fastigiata	chol	13
	Hypnea japonica	22-DC	14
	Ahnfeltia stellata	chol	13
	Chondrus crispus	chol	15,35
	C. giganteus	chol	34
	C. ocellatus	chol	34
	Gigartina stellata	chol	13
	Iridophycus cornucopiae	chol	34
	Rhodoglossum pulchrum	chol	33
Rhodymeniales	*Halosaccion ramentaceum*	chol, desmo	11
	Rhodymenia palmata	desmo, chol	11, 13, 15
	Coeloseira pacifica	chol	34
Ceramiales	*Ceramium rubrum*	chol	b
	Chondria dasyphylla	chol	b
	Laurencia pinnatifida	chol	13
	Polysiphonia nigrescens	chol	13
	P. lanosa (fastigata)	chol	13
	P. subtillisima	chol	b
	Rhodomelia conferoides	chol	11,15
	R. larix	chol	34
	Dasya pedicellata	chol	b
	Grinnellia americana	chol, desmo	b
	Rytiphlea tinctoria	camp or Δ^5 erg	16

[a]Desmo, desmosterol; chol, cholestenol; 22-DC, 22-dehydrocholesterol; camp, campesterol; Δ^5 erg, Δ^5-ergostenol.

[b]Doyle and Patterson, unpublished data.

been isolated from all major groups of living organisms. Cholesterol is the primary sterol of all higher animals; lower animals may contain cholesterol or a complex mixture of 27-, 28-, or 29-carbon sterols. The 28- and 29-carbon sterols found in primitive animals apparently result from their diet; none have been shown to be synthesized by animals.

In higher plants, β-sitosterol is commonly the principal sterol, although it is frequently accompanied by campesterol and stigmasterol. Sterols other than these are relatively rare in higher plants. In algae, however, the sterols are much more varied. Early work indicated that a complex mixture of sterols occurred in algae which was similar to the sterol mixtures of higher plants (5). A mixture of sitosterols was reported in several species of green algae (Chlorophyta). However, other species of Chlorophyta were reported to contain such widely-differing sterols as ergosterol (6), chondrillasterol (7), zymosterol (8), and fucosterol (5). While all publications showed that fucosterol was the major, if not the sole sterol of brown algae (Phaeophyta), they were in complete disagreement on the sterol content of red algae (Rhodophyta) (9). Most Japanese species of Rhodophyta contain cholesterol, while Rhodophyta from British waters contain sitosterol (9). These and many other questions concerning the occurrence and identity of sterols in algae have been resolved in recent years by a reexamination of the species in question using recently available methods of analysis.

TABLE II

Sterols of Brown Algae (Phaeophyta)

Order	Species	Major sterols[a]	References
Ectocarpales	*Pylaiella littoralis*	fuco	5
	Spongonema tomentosum (Ectocarpus tomentosus)	fuco	5
Sphacelariales	*Cladostephus spongiosus*	fuco	5
	Sphacelaria pennata (cirrosa)	fuco	5
	Stypocaulon scoparium	fcou	5
Dictyotales	*Dictyopteris divaricata*	fuco	18,39
	Dictyota dichotoma	fuco	5
	Padina arborescens	fuco	36
Chordariales	*Heterochordaria abietina*	fuco	36
Dictysiphonales	*Myelophycus caespitosus*	fuco	34
Laminariales	*Alaria crassifolia*	fuco	34,39
	Chorda filum	fuco	5
	Costaria costata	fuco, 24 MC	17, 41
	Eisenta bicyclis	fuco	17
	Laminaria angustata	fuco	36
	L. digitata	fuco, 24 MC	5,19
	L. faeroensis	fuco, 24 MC	19
	L. Hyperborea (cloustonii)	fuco	37
	L. japonica	fuco	36
	L. saccharina	fuco	37
Fucales	*Ascophyllum nodosum*	fuco	5,19,37
	Cystophyllum hakodatense	fuco	34
	Fucus gardneri	fuco	40
	F. evanescens	fuco, 24 MC	17,34,39
	F. diviarcarpus	fuco, 24 MC	41
	F. ceranoides	fuco	5
	F. serratus	fuco	37
	F. spiralis	fuco	37
	F. vesiculosis	fuco	37
	Halidrys siliquosa	fuco	5
	Pelvetia wrightii	fuco	17
	P. canalicalata	fuco	37
	Sargassum muticum	fuco	40
	S. confusum	fuco	39
	S. thunbergii	fuco, 24 MC	39
	S. ringgoldianum	fuco, 24 MC	39

[a]Fuco, fucosterol; 24 MC, 24-methylene cholesterol.

STEROLS OF RED ALGAE (RHODOPHYTA)

There are two probable reasons for the confusion concerning the sterol composition of Rhodophyta. First, when the earlier work with Rhodophyta was done (10), analytical techniques were not capable of separating and identifying closely-related sterols that were known to occur in other plants. Secondly, recent work has shown (11) that the sterol composition of a single species of Rhodophyta can be markedly different from sample to sample. It is not yet clear whether these differences are seasonal variations or due to factors related to the specific nutritional environments from which the samples were collected. In spite of the fact that we know the sterol composition of nearly 40 species of Rhodophyta, specific experiments have not been conducted to determine whether these sterols are synthesized by the algae or absorbed from their environment. It may be significant that *Porphridium cruentum*, a species of Rhodophyta which is cultured on a chemically-defined medium lacks sterols (12). The species of red algae examined for sterols are listed in Table I. The earliest sterol identifications have been omitted unless they have been substantiated by modern techniques of analysis. Cholesterol is the major sterol in most Rhodophyta; in many species, it is the only sterol detected. The major sterol of *Porphyra purpura* is desmosterol (13), but in *Porphyra sp.* (11), cholesterol predominates. The sterols of *Halosaccion ramentaceum* and *Rhodymenia palmata* are primarily desmosterol and cholesterol. In a given sample either may be found to predominate (11). *Hypnea japonica* contains 22-dehydrocholesterol as its major sterol (14), and several other species contain traces of this unusual sterol (11).

The following facts about the sterols of red algae are now apparent: (a) all Rhodophyta examined contain sterols except *Porphridium*

TABLE III

Sterols of Green Algae (Chlorophyta)

Order	Species	Major sterols[a]	References
Chlorococcales	*Scenedesmus obliquus*	chond, Δ^7-erg	7, 24, 42
	Chlorella vulgaris	chond, Δ^7-erg	24, 25
	C. fusca	chond, Δ^7-erg	b
	C. emersonii	chond, Δ^7-erg	b
	C. glucotropha	chond	b
	C. miniata	chond	b
	C. vannielii	erg	6, 26
	C. simplex	erg	26
	C. nocturna	erg	26
	C. sorokiniana	erg	26
	C. candida	erg	26
	C. ellipsoidea	porif, Δ^5-erg	25
	C. saccharophila	porif, Δ^5-erg	25
	Hydrodictyon reticulatum	spin	38
Ulvales	*Ulva lactuca*	28-iso	22
	U. pertusa	chol	39
	Enteromorpha linza	28-iso	23
	E. intestinalis	28-iso	22
	Monostroma nitidum	hali	23
Cladophorales	*Chaetomorpha crassa*	chol, 24-MC	39

[a]Chond, chondrillasterol; Δ^7-erg, Δ^7-ergostenol; erg, ergosterol; porif, poriferasterol; Δ^5-erg, Δ^5-ergostenol; spin, spinasterol; 28-iso, 28-isofucosterol; chol, cholesterol; hali, haliclonasterol; 24-MC, 24-methylene cholesterol.

[b]Patterson, unpublished data.

cruentum; (b) the predominant sterol of the great majority of Rhodophyta is cholesterol; and (c) with only one exception, the major sterols of the red algae have been C-27 sterols (Fig. 1). In some studies, traces of sterols tentatively identified as 24-methylene cholesterol, brassicasterol, stigmasterol, sitosterol or fucosterol have been reported (15,11). Gibbons, et al. (13) found no trace of C-28 and C-29 sterols in the Rhodophyta they examined and suggested that these algae could be incapable of alkylation at C-24 by S-adenosyl methionine. Some Rhodophyta apparently do contain C-28 and C-29 sterols; however, only in *Rytiphlea tinctoria* are they major sterols (16). It would be of interest to determine whether these sterols are synthesized by Rhodophyta or are absorbed from the environment.

STEROLS OF BROWN ALGAE (PHAEOPHYTA)

Since the earliest work of Carter, et al. (10), it has been recognized that fucosterol is the predominant sterol of Phaeophyta. Apparently, there is no exception to this rule (Table II). In one study (17), sargasterol, the C-20 isomer of fucosterol, was identified as the major sterol in *Sargassum ringgoldianum*, but this study did not make use of chromatographic methods now available to determine if the sterol is homogenous. More recent studies on this alga have not detected sargasterol (18,39). *S. ringgoldianum* apparently contains fucosterol as its primary sterol with small amounts of cholesterol, 24-methylene cholesterol and saringosterol (18). Recent work using gas chromatography has shown, without exception, the presence of small amounts of sterols other than fucosterol in Phaeophyta. The presence in brown algae of 24-methylene cholesterol and of desmosterol, which was tentatively identified in *Laminaria* (19), can be explained, since they could be biosynthetic precursors of fucosterol. Saringosterol (hydroxy-24-vinylcholesterol) (Fig. 2) has been identified in many recently-examined Phaeophyta (18,19,39). Knights has recently presented evidence that saringosterol arises from fucosterol by air oxidation (20).

Fucosterol is the major sterol in all brown algae examined. It is even more dominant in Phaeophyta than cholesterol is in Rhodophyta. Members of Phaeophyta are not closely related to any other algae (21). It may be phylogenetically significant, then, that fucosterol has not been frequently identified in other algal species.

STEROLS OF GREEN ALGAE (CHLOROPHYTA)

The sterols of Chlorophyta are much more complex than those of Phaeophyta and Rhodophyta. Early work indicated that the

TABLE IV

Sterols of *Chlorella*, Per Cent of Total Algal Sterol

Species	$\Delta^{5,7}$ Sterols		Δ^{7} Sterols			Δ^{5}-Sterols		
	Ergosterol	Unidentified $\Delta^{5,7}$ Sterols	Δ^{7}-Ergostenol	Chondrillasterol	Δ^{7}-Chondrillastenol	Δ^{5}-Ergostenol	Poriferasterol	Clionasterol
C. vannielii	76	24						
C. sorokiniana	75	25						
C. nocturna	66	34						
C. simplex	70	30						
C. candida	76	24						
C. protothecoides var. mannophila	35	65						
C. protothecoides var. communis	16	84						
C. ellipsoidea						28	56	16
C. saccharophila						30	60	7
C. vulgaris			25	65	10			
C. glucotropha			15	75	10			
C. fusca			27	59	12			
C. emersonii			28	62	9			
C. miniata			16	59	9			

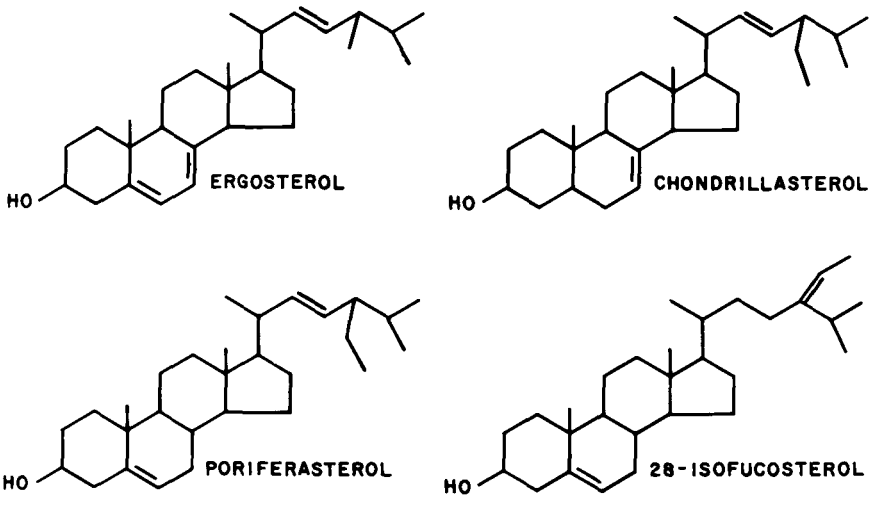

FIG. 3. Sterols of green algae.

sterols of Chlorophyta were similar to those of higher plants, that is, a complex mixture of sitosterols (5). However, a recent reexamination showed that *Ulva lactuca* and *Enteromorpha intestinalis* contain 28-isofucosterol (22), and not sitosterol as earlier work indicated (5). Other species which were shown to contain sitosterol in early work will probably be found to be in error also, since sitosterol has not been firmly identified in any green alga. These early examinations will not be considered here unless they have been substantiated by a recent reexamination. Due to the complexity of the sterol mixture in most Chlorophyta, many incorrect identifications have been made and are still being made. These mistakes may be largely avoided by use of modern techniques such as gas chromatography and thin layer chromatography and mass spectroscopy. Even with these techniques, identifications of these sterols are difficult because of the common occurrence, in Chlorophyta, of sterols with an asymmetric carbon atom at C-24. Just as a C-20 isomer of fucosterol (sargasterol) was once thought to occur in *Sargassum*, a C-20 isomer of campesterol has been tentatively identified in *Monostroma nitidum* (23). Thus the occurrence of haliclonasterol in *Monostroma* and spinasterol in *Hydrodictyon reticulatum* (38) must be regarded as tentative, since not enough data are available to determine the orientation at C-20 and C-24 (Table III). Cholesterol occurs in significant amounts in some species of Chlorophyta and may even be the primary sterol (39).

Considerable data are now available on the sterols of *Chlorella*, a unicellular green alga (Table IV). There is much variability in sterol composition within the members of this genus. Several species contain chondrillasterol and Δ^7-ergostenol with a smaller amount of Δ^7-chondrillastenol (24 and G.W. Patterson, unpublished data). Two species contain poriferasterol, Δ^5-ergostenol and clionasterol, and other species contain primarily ergosterol (26). It has been definitely established that all of these sterols contain alkyl groups at C-24

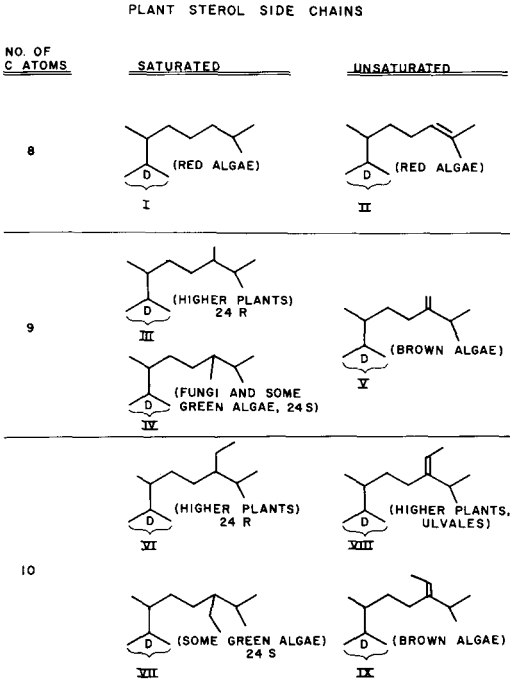

FIG. 4. Plant sterol side chains.

with the 24S configuration. The 24S configuration may be characteristic of green algal sterols in contrast to the 24R configuration of higher plant sterols (25) (Fig. 4). This certainly appears to be the case with *Chlorella*. Two strains of *Scenedesmus obliquus*, which were originally thought to contain different sterols (8), have now been found to contain chondrillasterol, Δ^7-ergostenol, and Δ^7-chondrillastenol in proportions similar to those *Chlorella* species containing these sterols (G.W. Patterson, unpublished data).

Some Chlorophyta contain cholesterol like Rhodophyta, some contain ergosterol like fungi, some contain 28-isofucosterol which occurs in higher plants and is similar to the fucosterol of Phaeophyta, and some contain sterols identical to those of higher plants (except for the configuration at C-24). Although most plant sterols have a nucleus identical to cholesterol, the structure of the side chain is quite variable. Thus the sterols of algae, particularly of the Chlorophyta, appear to have value for the systematist as a guide in taxonomy and phylogeny (Fig. 4).

STEROLS OF OTHER ALGAE

Only a few species of *Charophyta*, *Xanthophyta*, *Chrysophyta*, *Bacillariophyta* and *Euglenophyta* have been examined for sterols and most of this work was accomplished before identifications of sterols could be made with any confidence. Of the recent work, ergosterol has been identified in *Euglena gracillis* (27,28) and *Synura petersenii* contains cholesterol and sitosterol (29). Several species of *Ochromonas* have been examined for sterols. Although there was much early confusion concerning the sterols of *Ochromonas* species, it has been apparently established now that poriferasterol is the major sterol of *O. malhamensis* and *O. danica* (30). Although the sterol of *O. sociabilis* has been identified as stigmasterol (28), this sterol also seems to be poriferasterol. Recently, sterols have been isolated in very small amounts from the blue-green algae, *Phormidium luridum* (24-ethyl-Δ^7-cholestenol and 24-ethyl-$\Delta^{7,22}$-cholestadienol) (4), *Anacystis nidulans*, and *Fremyella diplosiphon* (cholesterol and 24-ethyl cholesterol) (3). The configuration at C-24 in the sterols of these algae is not yet known.

The biosynthesis of sterols in algae is just beginning to attract some interest. Cycloartenol has been shown to replace lanosterol as a biosynthetic precursor in *Fucus spiralis* (31), *O. danica* and *O. malhamensis* (30); algae may be quite similar to higher plants in the manner by which they synthesize sterols. When more data on sterol composition of algae are available, more progress may be made in the taxonomy and evolution of algae and higher plants. This information may also provide clues to the specific roles of sterols in plants.

ACKNOWLEDGMENT

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Functions of Sterols in Plants[1]

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ABSTRACT

Sterols have at least three functions in animals: they may act as precursors of other steroids, as hormones and as membrane components. The author advances the hypothesis that sterols have similar functions in plants.

INTRODUCTION

As a result of recent work on the biosynthesis and metabolism of steroids in microorganisms and higher plants (1,2), it is apparent that those compounds which occur in plants as well as in animals have essentially the same fate in all living organisms (3). This is not surprising to the biochemist, who has long ago come to recognize a certain unity of biochemical processes in cells of different origin. However, most people still find it difficult to accept the idea that the mode of action of compounds such as the steroid hormones should similarly exhibit a certain unity on the level of molecular biology. This reluctance is undoubtedly based on the obvious differences in gross manifestations of their biological activity. Yet for many compounds belonging to the vitamins, hormones, antibiotics and various drugs there is mounting evidence that the same chemical mechanism is at work, regardless of the origin of the cellular components upon which they act.

Sterols or their immediate precursors are the starting material for the biosynthesis of all other steroids. Thus, it is clear that one of their functions must be that of an inert stockpile of precursors that are readily convertible to biologically active compounds. More recently, a second function of sterols has come to light. Certain types of sterols may exhibit hormonal activity. A third type of function may be related to the fact that sterols are structural cell components. It has been postulated, e.g., that sterols play a part in cell permeability. Evidence for each type of function will be reviewed below.

BIOGENETIC FUNCTIONS

Cholesterol is a key intermediate in the biosynthesis of other steroids in plants as well as in animals (3). The steps leading to the formation of cholesterol are apparently the same for all living creatures, although certain intermediates may be more frequently encountered in the tissues of one organism than in those of another one. In animals, 7-dehydrocholesterol is known to be a precursor of cholesterol and of cholecalciferol (Fig. 1). Although these conversions have not been observed in plants so far, there can be no further doubt about the occurrence of cholecalciferol in plants (4).

Figure 1 shows some of the transformations of cholesterol we have observed in plants (1). Without degradation, the C_{27} sterol, cholesterol, is converted to C_{27} sapogenins, such as diosgenin, yamogenin and neotigogenin, to C_{27} alkaloids, such as tomatidine and solanidine, and to the C_{27} insect-molting hormone, ecdysterone. The dotted arrow pointing from solasodine indicates that its role as an intermediate has not been verified experimentally as yet. Likewise, there is no experimental verification of the postulated origin of ecdysterone from peniocerol via desoxyviperidone (5).

The C_{27} steroids formed from cholesterol are degraded by microorganisms and higher plants. Thus, e.g., *Mycobacterium phlei* converts diosgenin to the androstenedione and androstadienedione shown in Figure 1 (6). *M. phlei*, incidentally, also converts cholesterol to the same products (7,8). After the administration of radioactive cholesterol to *Lycopersicon pimpinellifolium*, we have isolated the labeled Δ^{16}-pregnenolone shown in Figure 1. Since tomatidine and neotigogenin were also labeled and since both of them yield Δ^{16}-pregnenolone when they are chemically degraded in the laboratory, we may assume that plants produce it by the same route. Although ecdysterone is known to be metabolized to the C_{21} steroid shown in Figure 1 by the blowfly (9), this reaction has not been demonstrated in plants so far. However, we may assume that rubrosterone (Fig. 1) is formed by such a process, because some plants, e.g., *Achyranthes rubrofusca*, contain both ecdysterone and rubrosterone.

The degradation of cholesterol in animals has long been known to involve the analogous 20,22-dihydroxycholesterol (Fig. 2) (3). We have now established that plants, like animals, metabolize cholesterol to pregnenolone. Pregnenolone, in turn, is the raw material from which plants synthesize other C_{21} steroids and cardiac

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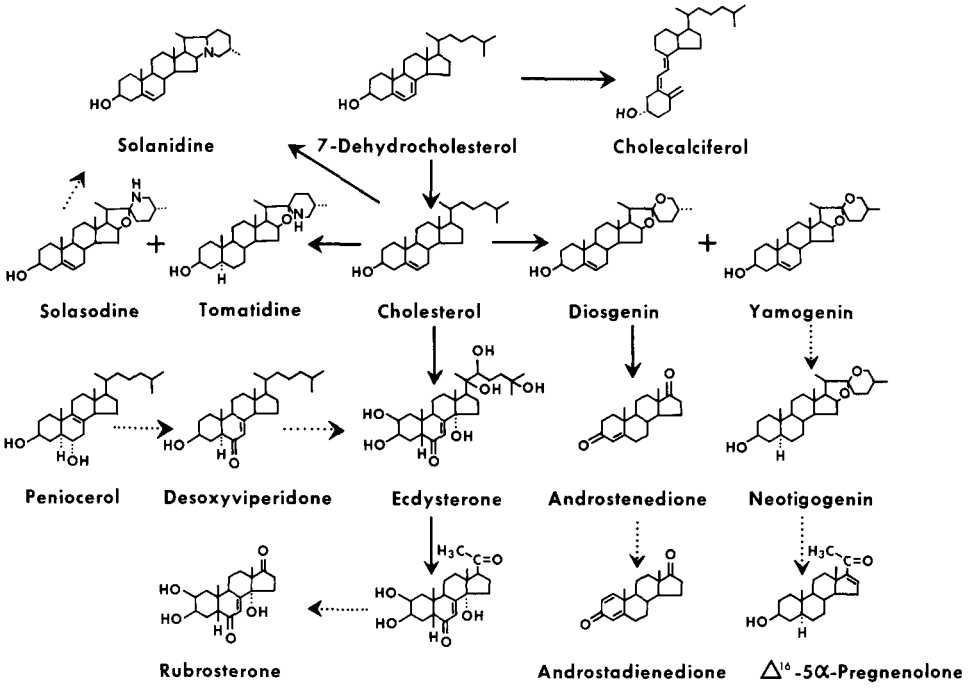

FIG. 1. Cholesterol metabolism.

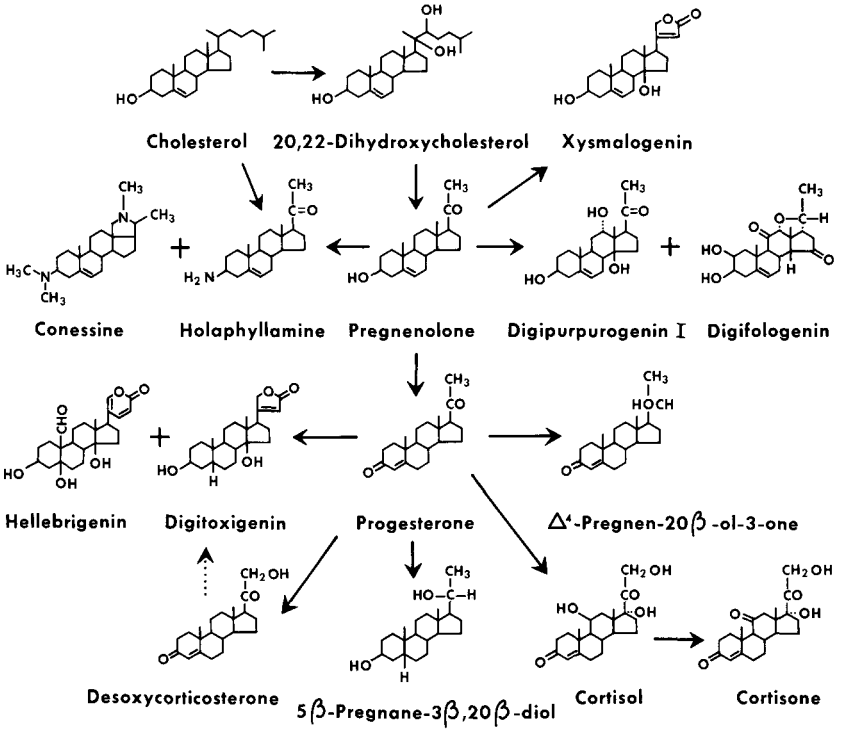

FIG. 2. Pregnenolone metabolism.

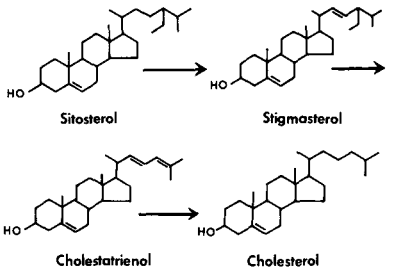

FIG. 3. Sitosterol metabolism.

aglycones (Fig. 2). The conversion of pregnenolone to various C_{21} alkaloids, such as holaphyllamine and conessine, has been observed in *Holarrhena*, and the formation of digitanols, such as digipurpurogenin and digifologenin, has been studied in *Digitalis*. Pregnenolone is directly converted to Δ^5-cardiac genins, such as xysmalogenin.

Progesterone is the most interesting metabolite of cholesterol in plants, because its specific endocrine effects in animals make its functions in plants seem rather perplexing. Progesterone has so far been isolated only from *Holarrhena floribunda* leaves and from apple seeds (10), but it undoubtedly occurs in many plants. Plant tissue cultures convert progesterone to Δ^4-pregnen-20β-ol-3-one, among other steroids. The acetate of that product has been isolated from the bark of *Khaya grandifoliola* (11). Progesterone is hydroxylated by animals and fungi at C-21 to form desoxycorticosterone and various other adrenocortical hormones, such as cortisol (Fig. 2) (3). The formation of desoxycorticosterone from progesterone has been observed in *Digitalis lanata* (12), and administered cortisol was converted to cortisone by *Mallotus paniculatus* (13). Two representatives of the cardiac genins formed from progesterone are shown in Figure 2: the C_{23} genin, digitoxigenin, and the C_{24} genin, hellebrigenin. The major biosynthetic pathway is said not to go through desoxycorticosterone (12).

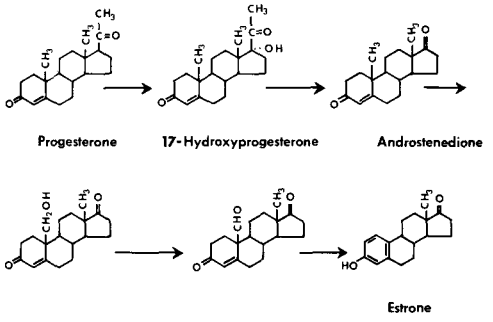

FIG. 4. Progesterone degradation.

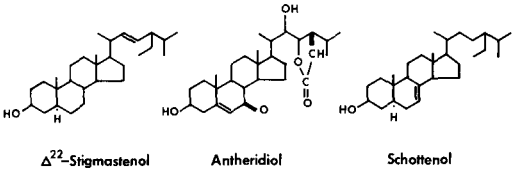

FIG. 5. C_{29} sterols.

The C_{28} and C_{29} sterols, which are much more abundant in plants than the C_{27} sterols, are synthesized from an unsaturated precursor of cholesterol. We have recently shown that the most common plant sterol, sitosterol, is dehydrogenated to stigmasterol in *Digitalis* plants (Fig. 3) (14). Insects are unable to synthesize sterols, but require them for the production of molting hormones (15). They have the ability to dealkylate stigmasterol by the route shown in Figure 3 (16). In our experiments with *Digitalis* we have observed that sitosterol is converted to progesterone (17) and to cardiac aglycones (18). Thus, sitosterol may function as a reserve supply from which plants can produce other sterols (perhaps including cholesterol) and progesterone.

In animals, progesterone is converted to estrone by the series of reactions shown in Figure 4 (3). Some microorganisms, such as *Nocardia restrictus*, have the ability to degrade various sterols to C_{19} 17-ketosteroids and further to estrone (19-21). Estrone has by now been isolated from a number of plant sources, such as the seeds of dates, pomegranates, and apples (22), from the pollen of date (23) and other flowers (24), and from moghat roots (23). It is quite likely that higher plants also synthesize estrone from sterols via progesterone and 17-ketosteroids.

HORMONAL FUNCTIONS

Sterols are not only biogenetic precursors of steroid hormones, but may have hormonal activity themselves. About 10 years ago, we were interested in the isolation of acrasin, the chemotactic hormone of a slime mold, *Dictyostelium discoideum* (25). Guided by the Shaffer assay, we determined that Δ^{22}-stigmastenol (Fig. 5), which the mold produces mainly during vegetative growth (26), and several related sterols promote the aggregation of slime mold cells. Difficulties with the assay have been an obstacle to further work on this problem.

Recently, the chemotactic hormone of a water mold, *Achlya bisexualis*, was identified and named antheridiol (Fig. 5) (27). It is a C_{29} sterol containing, in addition to the α,β-unsatu-

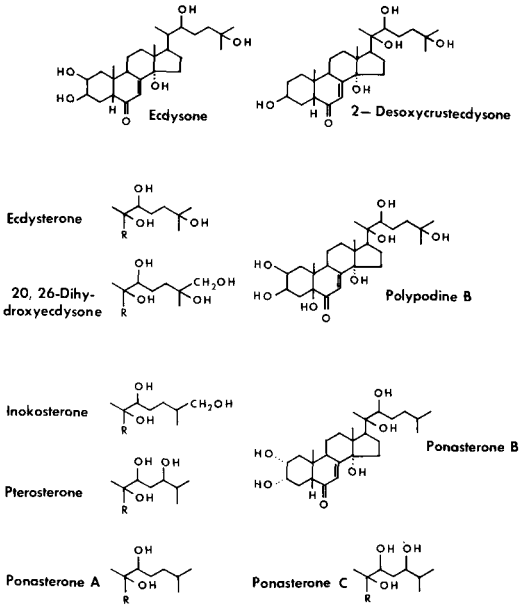

FIG. 6. C_{27} insect-molting hormones.

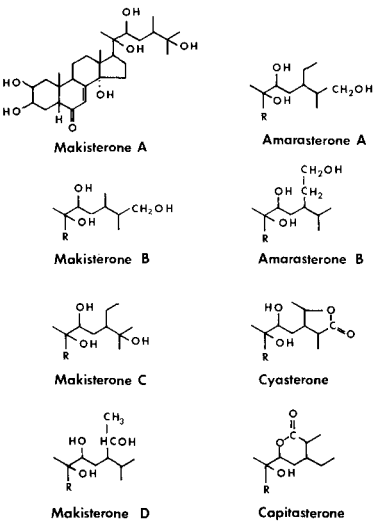

FIG. 7. C_{28} and C_{29} insect-molting hormones.

rated carbonyl group commonly found in steroid hormones, the α,β-unsaturated γ-lactone ring characteristic of the cardenolides. It seems likely that Δ^{22}-stigmastenol is not the acrasin we have been looking for, but a precursor of a steroid hormone analogous to antheridiol. Cyclic AMP has recently been found to have acrasin activity (28). This substance is a widely distributed second messenger, known to mediate the action of a large assortment of hormones (29).

A great variety of sterols with insect-molting hormone activity occurs in plants (Fig. 6 and 7) (5). All of these compounds contain an α,β-unsaturated carbonyl group and a 14α-hydroxyl group. Although the concentration of these sterols is much higher in plants than in insects, they remained unrecognized until very recently, partly because they are water-soluble and partly because there was no bioassay available earlier. The function of this group of sterols in insects is now quite clear. In fact, it constitutes the best example of the mechanism of action of steroid hormones (30).

Whether insect-molting hormones in plants have any effects, favorable or unfavorable, on the insects normally feeding on these plants is still debatable (5). Most authors like to look upon these hormones as insect hormones and do not expect them to affect the plants that contain them (31). Except for a report of gibberellin activity of ecdysone in dwarf peas (32), there is no indication in the literature that the effect of insect-molting hormones on plants has been tested systematically. The reported effects on animals, such as the stimulation of protein synthesis in the liver (33) and the aggregation of insect cells in tissue cultures (34) suggest plant systems worthy of exploration.

In insects, the molting hormones mediate the photoperiodic stimulus to induce metamorphosis. It is conceivable that morphological changes in plants that are known to be under photoperiodic control are similarly regulated. Some years ago, we discovered that the administration of inhibitors of cholesterol biosynthesis to short-day plants suppresses their flowering response to dark-treatment (35). In spite of occasional reports of floral induction by sterols (36-38), the role of steroids in plant morphogenesis is still a matter of conjecture. With the advent of a reliable bioassay (39) progress in this important area of research should now be rapid.

In view of the importance of sterols to microorganisms (40), the evidence for their presence has been reinvestigated. The argument is now settled and it may be taken for granted that at least some bacteria (41), as well as blue-green algae (42,43), contain sterols. However, some microorganisms may lack the ability to synthesize the steroid nucleus (44,45). In many cases growth inhibition may be produced by inhibitors of sterol biosynthesis and repaired by the administration of sterols (46-48). The structural specificity of sterols having growth-promoting activity is rather low. Various Δ^5-C_{27}, -C_{28} and -C_{29} sterols are effective (49).

Phytopathogenic fungi belonging to the genera *Pythium* and *Phytophthora* require exo-

genous sterols for reproduction (50-54). Again, all Δ^5-sterols tested so far are active (55,56), perhaps because they are metabolized to the same hormone (57). However, estradiol, one of the final products of sterol metabolism, counteracts the sterol-induced sexual reproduction (58). Inhibitors of sterol biosynthesis prevent sexual differentiation in *Cochliobolus carbonum*, and sterols repair it (59). The sexual hormones in *Hansenula wingei* (60) and in *Saccharomyces cerevisiae* (61) are said to be sterols.

OTHER FUNCTIONS

Many authors have now studied the relations between the selective toxicity of polyene antibiotics for fungi and their sterol composition (62-66). The conclusion may be drawn that these antibiotics inhibit growth and reproduction by somehow interacting with the sterols in the cell membranes (67,68) and permitting material to leak out of the fungal cells (69). Thus, the sterols in the fungi make them susceptible to polyene antibiotics (70). The antagonism between sterols and polyene antibiotics permits the reversal by cholesterol of the antibiotic-induced growth inhibition (71) and the inhibition by these antibiotics of cholesterol-induced oogenesis (72).

Other reported effects of sterols include the increased temperature tolerance in *Pythium* (52,73,74), which may well be related to the role of sterols in cell permeability phenomena. Sterols are required for mitochondrial function. Thus, inhibition of sterol synthesis in yeast reduces respiratory competency, which can be restored by the addition of ergosterol (75). Anaerobically grown yeast synthesized mitochondrial enzymes faster, if it was supplied with ergosterol (76). Under growth-limiting oxygen tension the obligate aerobe *Candida parasilosis* showed a higher cytochrome concentration when it was grown in the presence of ergosterol (77). *C. cylindracea* was reported to require a sterol for lipase production (78).

The dependence of sterol-requiring saprophytes on sterol-containing host plants has a parallel in the dependence of insects on certain sterol-producing organisms. This has been documented in the case of *Drosophila pachea*, a fly which lives on the senita cactus (79). The cactus supplies schottenol (Fig. 5), which the fly apparently requires for the partial synthesis of a molting hormone.

Many of the products of sterol metabolism in plants, such as the C_{27} sapogenins and alkaloids, steroidal antibiotics and cardiac glycosides, may have regulatory as well as ecological functions. They may be involved in regulating the life processes of plants or help them to survive in the face of their numerous foes, such as fungi, insects and higher animals. Other products of sterol metabolism, such as progesterone, rubrosterone and estrone may serve hormonal functions in the plants that produce them (80). Be this as it may, the functions of sterol metabolites are outside of the scope of this review.

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Some Pathways and Mechanisms in Lanosterol-Cholesterol Conversion in Mammalian Tissues

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ABSTRACT

Sterol precursors of cholesterol with a double bond in the lateral chain are present in normal human brain and glioblastoma. Biosynthetic experiments with labeled mevalonate show that the unsaturated intermediates are preferentially labeled when compared with the corresponding saturated sterols. The biological role of sterols containing an 8(14) double bond is discussed.

INTRODUCTION

The rate of cholesterol biosynthesis varies in mammalian tissues with age and degree of maturation. Immature brain has a high rate of acetate and mevalonate conversion into cholesterol while adult brain has a very low level of biosynthesis (1,2). Liver sterol synthesis, however, which has been studies most, is very active, especially in mature animals, and may be influenced physiologically by many factors (3) including diet, hormones and ions.

After cyclization of squalene to lanosterol, depending on the sequence of structural modifications of lanosterol leading to cholesterol, the formation of a large number of intermediates may be involved (4). There are indications of a preference for either one route or another by different tissues. For example, liver enzyme systems may saturate the Δ^{24}-double bond in the lateral chain at the lanosterol level, and later, at desmosterol level (5). In brain, large amounts of desmosterol are present during development (6-8); its disappearance from mature brain (9) and its efficient labeling (10) indicate an involvement of Δ^{24} intermediates.

This report attempts to demonstrate the existence of preferential pathways of cholesterol biosynthesis in tissues such as brain during normal maturation and pathological growth, as well as in mature nervous tissue and normal liver. This is achieved through the identification of the sterols isolated from both animal and human tissues, determination of their specific activity in biosynthetic experiments, and utilization for biosynthesis of some of the suspected precursors found in the tissues (11).

[1]One of 12 papers to be published from the "Sterol Symposium" presented at the AOCS Meeting, New Orleans, April 1970.

EXPERIMENTAL PROCEDURES

Human Brain and Brain Tumors

For the in vivo experiments, selected patients were injected intravenously with 200 μC of (DL) [2,$^3H_2$]- mevalonic acid lactone (supplied by the Radiochemical Centre Amersham, U.K., specific activity 100 mC/mmole) 10 hr before surgery. For in vitro experiments, human brains and brain tumors were obtained from patients during neurosurgical operations and were kept in cold buffer up to the time incubation started. Large necrotic areas and hemorrages were removed. The minced tissues were incubated in Krebs-Ringer phosphate buffer (10 ml/g tissue). The incubations were carried out for 2 hr in O_2 atmosphere at 37 C after addition of 2 μC/g tissue of DL-[2^{14}C]-mevalonic acid lactone (supplied by the Radiochemical Centre Amersham, U.K., specific activity 5 mC/mM). The tissues were saponified in ethanolic KOH, and the sterols were extracted and identified. The methods have already been described in detail (12,13).

Identification of Specific Sterol Precursors

The sterol mixtures from the unsaponifiable fractions were acetylated and then transferred to a chromatographic column packed with Silica Gel G-Celite-$AgNO_3$ (14). The complex mixture of biological sterols present in brain and brain tumors are not resolved completely, because the column separation is based more on number and position of double bonds than on molecular weight. The sequence of the chromatographic elution of the sterols is as follows: the least polar sterol, cholestanol, the Δ^8 series, cholesterol (Δ^5), sterols with two double bonds, [one in the lateral chain (Δ^{24}) and one in the nucleus (Δ^8 and Δ^7)], desmosterol ($\Delta^{5,24}$), Δ^{14} sterol and sterols with conjugated double bonds ($\Delta^{8,14}$ and $\Delta^{5,7}$). The more oxygenated sterols are eluted last. The homologous sterols with 30, 29, 28 and 27 carbon atoms may not be completely separated. Each subfraction has been resolved using quantitative gas liquid chromatography (GLC) (14) and the structure of the single sterols identified using a

TABLE I

Sterols Identified in Adult Human Brain and Glioblastoma

Sterol	Brain		Glioblastoma	
	Amount of total sterols, %	Amount μg/g tissue	Amount of total sterols, %	Amount μg/g tissue
C_{30} Δ^8	0.003	0.596	ND	ND
C_{29} Δ^8	.030	5.642	0.002	1.04
C_{28} Δ^8	.020	3.857	.023	13.76
C_{27} Δ^8	.082	15.480	.120	7.13
C_{30} $\Delta^{8,24}$	.004	0.708	ND	ND
C_{29} $\Delta^{8,24}$	.019	3.588	.007	4.34
C_{28} $\Delta^{8,24}$	.016	3.064	.046	27.81
C_{27} $\Delta^{8,24}$	.011	2.157	.005	3.20
C_{27} $\Delta^{5,24}$	.020	3.838	1.186	712.00
C_{27} Δ^5	99.090	18,660.000	95.632	57,423.62
C_{27} Δ^{14}	0.020	3.835	ND	ND
C_{27} Δ^5 (7C=0)	.009	1.626	ND	ND
C_{27} Δ^0	.291	54.750	0.178	106.80
Unidentified and partially identified compounds	.384	72.380	2.909	1,746.72

[a]ND, none detected.

TABLE II

Sterol Biosynthesis in Human Brain and Human Glioblastoma

Sterol	Brain		Glioblastoma	
	Per cent of total sterols	Per cent of sterols radioactivity	Per cent of total sterols	Per cent of sterols radioactivity
In Vitro				
Δ^8 series	0.132	5.10	0.146	0.10
$\Delta^{8,24}$ series	0.050	12.00	0.058	28.00
$\Delta^{5,24}$ (Desmosterol)	0.020	2.11	1.186	20.71
In Vivo				
Δ^8 series		Trace		0.06
$\Delta^{8,24}$ series		9.31		4.80
$\Delta^{5,24}$ (Desmosterol)		11.41		11.74

TABLE III

Enzymatic Conversion of
[2-$^3H_2$]-4.4-Dimethyl-5α-cholest-8(14)-en-3β-ol[a]

Radioactive II, μg	Unlabelled III, μg	Unsaponifiable, dpm	Cholesterol		III	
			dpm	% conv.	dpm	% conv.
62[b]	---	1,110,000	204,000	18.50	ND[c]	ND
62[b]	1,500	717,000	2,100	0.30	ND	ND
81[d]	---	628,000	97,500	15.70	6,540	1.04
81[d]	1,500	563,000	10,200	1.80	6,930	1.23
58[e]	---	992,000	115,000	11.60	5,880	0.59
58[e]	1,500	900,000	900	0.10	13,400	1.49

[a]For each experiment 3 flasks containing 10 ml of 10,000 x g liver homogenate were used. The flasks were combined after the incubation.

[b]Specific activity 4.60 μC/μ mole.

[c]ND, nondetermined.

[d]Specific activity 3.03 μC/μ mole.

[e]Specific activity 4.95 μC/μ mole.

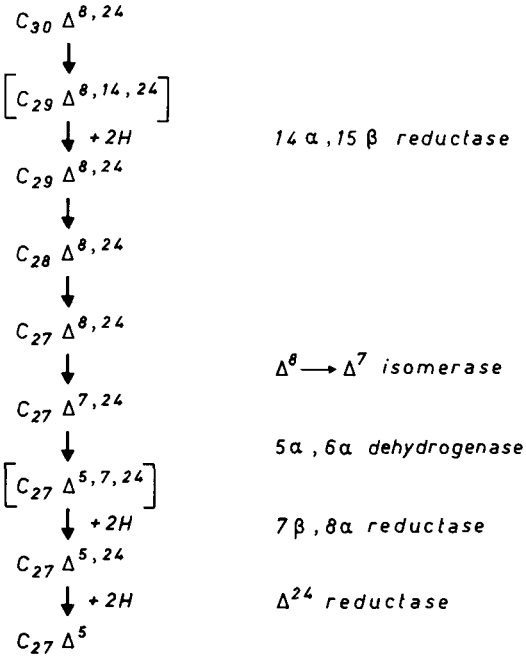


FIG. 1. Proposed pathway of cholesterol biosynthesis in nervous tissue.

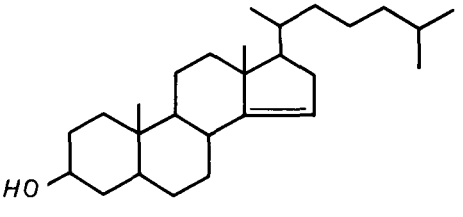
combination of gas chromatography and mass spectrometry (13,15). An LKB 9000 gas chromatograph-mass spectrometer was used. The identification of the biological sterols was obtained by comparison with authentic sterols.

Radioactivity Determinations

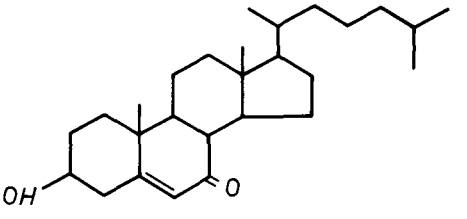
Each sterol sample was dissolved in 0.5 ml benzene and counted for radioactivity with a Standard Scintillation mixture (PPO 4 g, POPOP 100 mg, toluene 1000 ml) using a liquid scintillation spectrometer (Tri-Carb Packard, 3000 Series). Disintegrations per minute (dpm) were calculated with the aid of internal standards. The counting efficiency was 90% for ^{14}C and 32% for ^{3}H.

Rat Liver

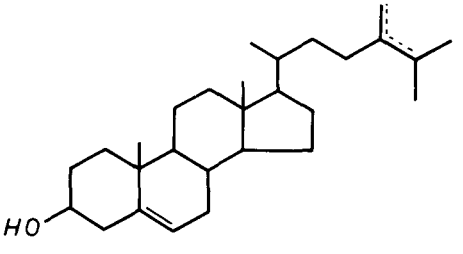
When liver was used as the enzyme source, livers of male Sprague-Dawley rats weighing 200 g were rapidly removed, repeatedly washed in buffer, and homogenized according to Bucher and McGarrahan (16) with 2.5 vol of 0.1 M K-phosphate buffer containing 0.03 M nicotinamide and 0.006 M $MgCl_2$. The total homogenate was centrifuged for 10 min at 10,000 x *g* supernatant was used for the incubations. After being synthesized (Fiecchi) et al. unpublished data) (2,$^{3}H_2$]-4,4-dimethyl-5α-cholest-8(14)-en-3β-ol was incubated with rat liver homogenate, in oxygen atmosphere for 1 hr at 37 C. In trap experiments, unlabeled 4,4-dimethyl-4α-cholesta-8,14-dien-3β-ol dissolved in Tween 80 was preincubated with the liver homogenate for 15 min under nitrogen atmosphere. The labeled 4,4-dimethyl-5α-cholest-8(14)-en-3β-ol was added afterwards, and the incubation was continued under oxygen for 1 hr.



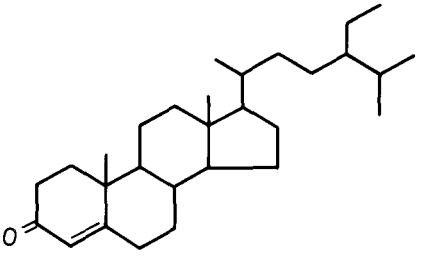
5α-CHOLEST-14-EN-3β-OL



7-KETOCHOLESTEROL



24-METHYLENE-CHOLESTEROL
or 24-METHYL-DESMOSTEROL



β-SITOSTENONE

FIG. 2. Structures of sterols out of the known pathway of cholesterol biosynthesis, identified in human brain.

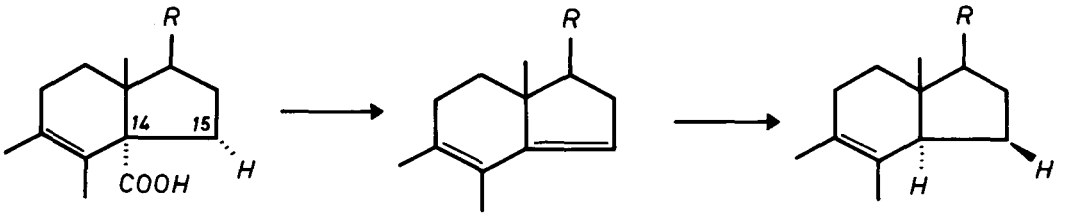


FIG. 3. Role of 4,4-dimethyl-5α-cholesta-8,14-dien-3β-ol in cholesterol biosynthesis.

The unsaponifiable fractions were acetylated with acetic anhydride in pyridine and, after dilution with unlabeled 4,4-dimethyl-5α-cholesta-8,14-dien-3β-ol acetate, the acetylated sterols were separated by silver nitrate Silica Gel G-Celite column chromatography; cholesterol acetate and 4,4-dimethyl-5α-cholesta-8,14-dien-3β-ol acetate were further purified as previously reported (17).

RESULTS

The results obtained by analysis of samples of adult human brain and a glioblastoma are summarized in Table I.

In adult human brain, cholesterol represents 99% of total sterols; in glioblastoma, it is only 95%. The major sterol in the glioblastoma, after cholesterol, is desmosterol (1.19%). Normal brain, however, has a very low concentration of this sterol which does not exceed that of the other sterol precursors. The sterols with 30, 29, 28 and 27 carbon atoms and with double bonds in 8 or in both 8 and 24 positions, are present in small amounts both in the brain and in the tumor. Cholestanol (saturated C_{27}), believed to be a catabolic product of cholesterol, is quantitatively the second sterol in the brain and the third one in the tumor.

A small percentage of sterols (0.39%) in the brain and a larger one in the tumor (2.90%) is represented in part by oxygenated products of cholesterol catabolism and in part by partially identified sterols with one and two double bonds. There are only two major variations in sterol composition of the normal human brain and the brain tumor: the amount of desmosterol is 10 times higher and the amount of the catabolic products 6 times higher in the tumor than in the brain. The sterols identified in human brain (12) and in brain tumors are essentially the same as found in the developing brain of the chick embryo and rat (10). The major difference is the lack of $\Delta^{7,24}$ sterols in the first two tissues; however, we cannot completely exclude their presence.

The distribution of radioactivity is of particular interest. In adult brain and glioblastoma the highest percentage of radioactivity was found in the $\Delta^{8,24}$ series (12% in normal, 28% in glioblastoma, against 5.1% and 0.1% respectively in the saturated Δ^{8} series). The specific activity is even higher because the total amounts of $\Delta^{8,24}$ sterols are lower than the corresponding Δ^{8} precursors (Table II). The results obtained in vitro incubation of brain and glioblastoma were confirmed by in vivo experiments. The incorporation of labeled mevalonate in brain was 9.3% in the $\Delta^{8,24}$ series, 11.4% in desmosterol and only traces were found in the Δ^{8} series. Similar patterns were observed in the glioblastoma-bearing patients where the tumor removed after the in vivo administration of labeled mevalonate showed 4.8% of radioactivity in the $\Delta^{8,24}$ series, 11.7% in desmosterol and, again only traces in the Δ^{8} series.

The data are consistent with the hypothesis that the preferential pathways for cholesterol biosynthesis in mature, as well as in pathological human brain, lead to the formation of $\Delta^{8,24}$ and $\Delta^{5,24}$ precursors, suggesting a rate-limiting step at the level of the Δ^{24} reductase in brain (Fig. 1). Among the sterols detected in adult human brain the following compounds have been identified: 5α-cholest-14-en-3β ol, 7-ketocholesterol, β-sitostenone and a sterol tentatively identified as 24-methylencholesterol or 24-methyldesmosterol (Fig. 2). The presence of 5α-cholest-14-en-3β-ol in nervous tissue was particularly interesting because this further supports the demonstation of the biological significance of sterols containing a double bond at 14-15 (20).

In our previous investigations the loss of the 15α hydrogen of lanosterol during the removal of the 32 methyl group was demonstrated while the formation of a 32 carboxyl group which is later eliminated as carbon dioxide was suggested by others (20).

Later it was demonstrated that an 8,14-diene sterol should also be considered a biological precursor of cholesterol. This is confirmed by: (a)the transformation of 4,4-dimethyl-5α-cholesta-8,14-dien-3β-ol (III) into 4,4-di-

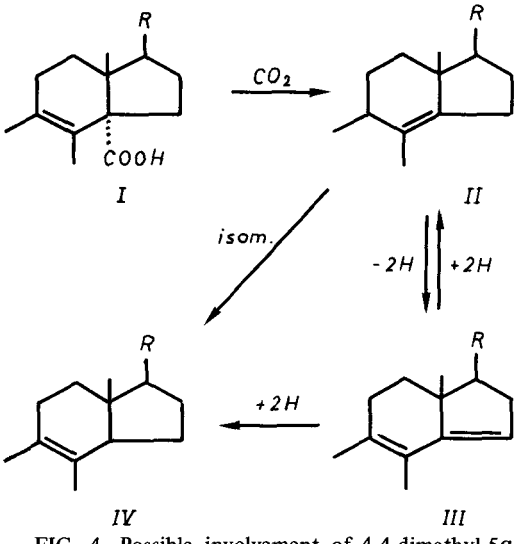


FIG. 4. Possible involvement of 4,4-dimethyl-5α-cholest-8(14)-en-3β-ol in cholesterol biosynthesis.

methyl-5α-cholest-8,(9)-en-3β-ol (IV) (Fig. 3) (18); (b)the blockade of the transformation of radioactive dihydrolanosterol into cholesterol by addition of a pool of unlabeled 4,4-dimethyl-5α-cholest-8,14-dien-3β-ol, used as a biological trap (17); and (c)the recently-announced isolation of a sterol present in human meconium, identified as 4,4-dimethyl-5α-cholesta-8,14-dien-3β-ol (21).

Our investigations deal with the position of this compound in the reaction sequence of lanosterol-cholesterol conversion. Two possible routes are outlined in Figure 4. The compound (III) could be formed from 4,4-dimethyl-5α-cholest-8(9)-en-3β-ol 32-carboxylic acid (I) through 4,4-dimethyl-5α-cholest-8(14)-en-3β-ol (II), involving a decarboxylation followed by a dehydrogenation. The same compound (III), however, could also be the immediate precursor of II through a single hydrogenation. Compound II could be isomerized into 4,4-dimethyl-5α-cholest-8(9)-en-3β-ol (IV).

The previous experimental evidence indicates that compound III cannot be a precursor of II because it is not transformed into II in N_2 atmosphere (18). In addition to this finding, Lee et al. (22) demonstrated that compound II cannot be transformed into compound IV under N_2.

Stimulated by the interesting observation by Lee et al., and Fried et al., (23) that compound II is readily transformed into cholesterol under O_2, we have tested whether this is indeed a precursor of a 8,14 diene sterol (III). We have utilized compound II, chemically synthesized and labeled at position 2 with tritium and incubated with liver homogenate, confirming that in the presence of oxygen there is a conversion into cholesterol. A trap of 4,4-dimethyl-5α-cholesta-8,14-dien-3β-ol (III) greatly reduces the incorporation into cholesterol without a radioactivity increase in 4,4-dimethyl-5α-cholesta-8,14-dien-3β-ol (Table III). These results indicate that 8(14)ene is not intermediate of the conversion of III into IV).

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Potential Function of Cholesterol in Blood Coagulation: Amplification of Phospholipid Thromboplastic Activity

ABSTRACT

Recalcification tests have revealed a two- to threefold increase in the thromboplastic activity of recombined phospholipid fractions from chicken erythrocyte lipids on supplementation with the original concentration of cholesterol. An optimal four- to fivefold increase was obtained at higher levels. Cholesterol esters had no such effect and clotting times with Russell's viper venom were not similarly affected.

Ever since the early observations were made on the accelerating effects of tissue and blood lipids on blood coagulation (1-6), the chemical nature and effects of the active principles have undergone investigation (7-9). Although these problems have not yet been solved to the satisfaction of all investigators, thromboplastic activities of lipids have always been associated with phospholipid fractions, and no role has hitherto been attributed to less polar components of crude lipid extracts.

In attempts to account for the thromboplastic activities of sonicated chicken erythrocyte lipids in studies of recombinations of phospholipid classes separated by column chromatography, thin layer chromatography (TLC), or both, we consistently failed to recover more than 50-55% of the original activity as determined by recalcification (Ca-test) of citrated chicken plasma. In contrast, activity recoveries in excess of 90% were obtained in clotting tests with Russell's viper venom (Rvv-Ca-test). The lipid requirement of reaction steps which are limiting for the overall coagulation rate thus appeared to differ from that of the reactions triggered by Rvv. Participation of a nonphospholipid component in an early stage in intrinsic blood coagulation seemed likely.

To characterize the nonphospholipid component, crude chicken erythrocyte lipids, extracted by chloroform-methanol 2:1 and 1:2,

TABLE I

Effect of Plasma Clotting Times of Fractions of Chicken Erythrocyte Lipids[a]

Concentration of lipid[b]	Clotting trigger	Recombined fractions, sec: 1-8 (neutral + polar)	Recombined fractions, sec: 2-8 (polar only)
c/2	$CaCl_2$, 25 mM	136	178
c/4		169	217
c/8		207	277
Buffer control: >1400 sec			
c/8	Rvv in $CaCl_2$,	8.3	8.2
c/16	25 mM (1:15000)	10.0	9.8
c/32		12.0	11.8
Buffer control: 15.6 sec			

[a]Averages in seconds of duplicate and triplicate runs with a Coagulation Analyzer, Model CA-550, Biodynamics, Inc. Test system: 0.1 ml each of chicken citrated plasma, lipid emulsion and clotting trigger.

[b]Stock emulsions obtained by sonication of fractions from 0.4 mg of crude erythrocyte lipids in 4 ml of buffered saline, with original concentration diluted by the figures given.

TABLE II

Effect of Graded Concentrations of Cholesterol on Plasma Recalcification Times of Recombined Phospholipid Fractions[a]

Concentration of lipid	Sephadex fraction 1, sec	Cholesterol phospholipid ratios[b]							
		TLC fraction of erythrocyte cholesterol				Pure cholesterol standard			
		0	1/2	1	2	1	2	4	8
c/2	134	160	153	135	114	134	112	112	114
c/4	158	192	182	158	131	157	128	127	133
c/8	188	226	215	188	155	186	150	150	158
Buffer control: >1200 sec									

[a]Previously separated by one-dimensional TLC using chloroform-methanol-NH_3 (28%), 65:35:5.
[b]Relative to the ratio in crude erythrocyte lipids.

were purified by chromatography on Sephadex G-25 (10). More than 90% of the original thromboplastic activities, as measured by Ca- and Rvv-Ca-tests, were consistently recovered in the first Sephadex G-25 fraction of the chicken erythrocyte lipids which contained mainly neutral lipids, phospholipids and glycolipids. Subsequent eluates containing possible traces of gangliosides and nonlipid impurities had no activity of their own and did not increase the activities of the first fraction in recombination experiments.

The active Sephadex Fraction 1, after TEAE-cellulose column chromatography (11) yielded no single fraction having appreciable thromboplastic activity, but recoveries of activity in excess of 90% were obtained on recombination of all TEAE-cellulose fractions, thus supporting the view that lipid thromboplastic activity is due to a synergistic action of separate lipid classes (12-16). Thus, an assessment of the contribution of each of the fractions could be obtained only by systematic exclusion experiments.

As shown in Table I, omission of the neutral lipids (Fraction 1) from a mixture of all fractions (Nos. 1 to 8) decreased thromboplastic activity by more than 50%, as measured by the recalcification (Ca-test), whereas the activity with the Rvv-Ca-test was unchanged or slightly increased.

The activity-amplifying effect of the neutral lipids was found to be associated with the cholesterol fraction separated by silica gel TLC (17).

As shown in Table II, the results obtained with the TLC fraction of erythrocyte lipids and equivalent concentrations of a pure cholesterol standard were identical and an apparent plateau representing a four- to fivefold increase in activity was observed at cholesterol-phospholipid ratios of two to eight times that present in erythrocyte lipids.

Equivalent concentrations of cholesterol esters as isolated from chicken plasma did not change the activity of recombined erythrocyte phospholipid fractions, making it apparent that the amplifying effect was restricted to free cholesterol.

To the best of our knowledge, this is the first unequivocal demonstration of a potential role of free cholesterol in blood coagulation, thus possibly linking blood hypercoagulability and increased risk of thromboembolic disease with elevated levels of cholesterol. A more detailed report of these and related results is in preparation.

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Hepatotoxicity and Lipid Metabolism-IV Structure and Molecular Species of Hepatic Triglyceride in Rats Treated With Carbon Tetrachloride

ABSTRACT

Effects of carbon tetrachloride (CCl_4) on the positional distribution of fatty acids and on the molecular species of hepatic triglyceride in female rats were studied. Six hours after a dose of CCl_4, the percentages of palmitic and oleic acids were increased at the 1 and 2 positions respectively in comparison to the control, while linoleic acid was decreased at the 3-position. These changes were not remarkable 20 hr after CCl_4. CCl_4 treatment generally caused increases in less unsaturated species of triglycerides (S_2M, SM_2 and S_2D) and decreases in more unsaturated species (SD_2 and more polyunsaturated). These data suggest that CCl_4 induces specific alteration in the metabolism of triglycerides in the liver.

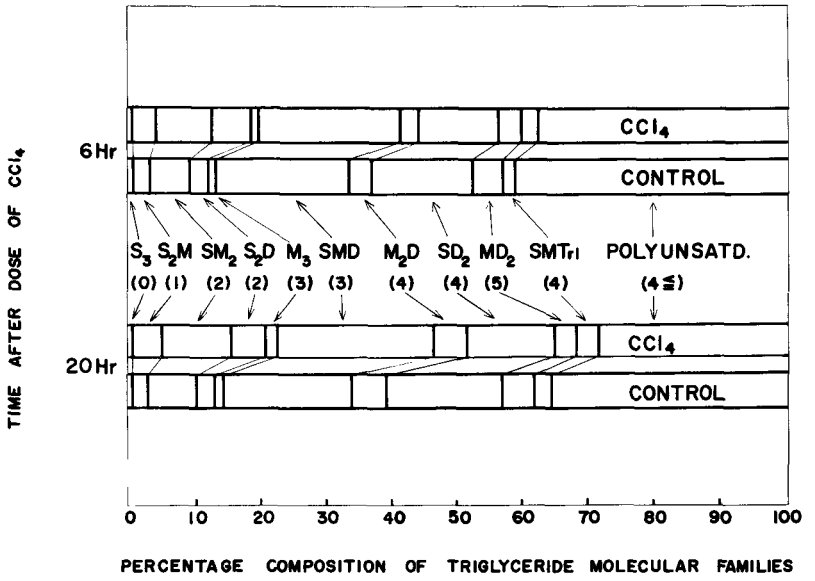

FIG. 1. Molecular species analyses of triglycerides from CCl_4 treated and control rats. Values are means of two independent experiments. Pooled liver from five rats per group. Numbers in parentheses represent total degree of unsaturation.

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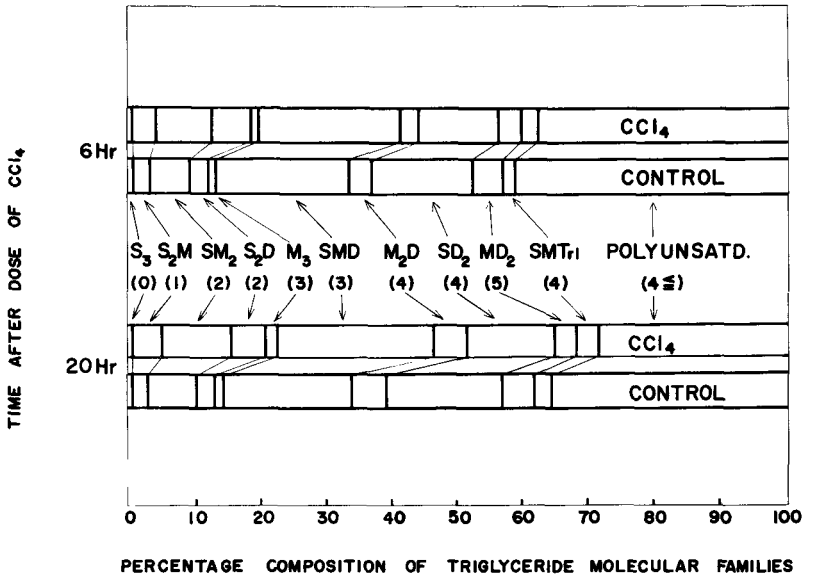

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TABLE I

Effect of CCl_4 on the Positional Distribution of Fatty Acids in Liver Triglyceride[a]

Fatty acids	Distribution, mole %					
	1 Position		2 Position		3 Position	
	CCl_4	Control	CCl_4	Control	CCl_4	Control
6 hr						
16:0	27.4 ± 0.9[c]	22.6 ± 1.0	0.9 ± 0.1	1.2 ± 0.1	4.3 ± 0.8[d]	2.4 ± 1.0
16:1	1.1 ± 0.1	1.4 ± 0.4	0.6 ± 0.2	0.4 ± 0.1	1.1 ± 0.4	0.1 ± 0.1
18:0	3.1 ± 0.5	4.0 ± 0.7	0.2 ± 0.1	0.3 ± 0.0	Trace	Trace
18:1	1.5 ± 0.1[b]	4.9 ± 0.7	11.3 ± 0.4[b]	9.1 ± 0.1	10.7 ± 1.2	7.7 ± 0.9
18:2	Trace	Trace	18.1 ± 0.3	19.8 ± 0.4	12.8 ± 0.4[c]	18.5 ± 1.0
18:3	0.1 ± 0.1	0.3 ± 0.2	0.8 ± 0.2	0.9 ± 0.2	2.3 ± 0.2	2.3 ± 0.3
20:4	Trace	Trace	1.3 ± 0.1	1.5 ± 0.2	2.0 ± 0.5	2.3 ± 0.5
20 hr						
16:0	22.8 ± 1.0	20.7 ± 0.9	1.0 ± 0.0	1.2 ± 0.1	6.5 ± 1.4	5.6 ± 1.4
16:1	1.9 ± 0.3[d]	1.0 ± 0.1	1.2 ± 0.1	0.7 ± 0.1	2.7 ± 0.4[b]	0.8 ± 0.2
18:0	1.8 ± 0.4	2.1 ± 0.1	0.2 ± 0.0	0.1 ± 0.1	Trace	0.5 ± 0.8
18:1	5.1 ± 0.8[d]	7.4 ± 0.3	10.9 ± 0.6	9.5 ± 0.3	7.4 ± 0.8	5.9 ± 0.7
18:2	1.0 ± 0.5	1.6 ± 0.7	17.8 ± 0.6[d]	20.0 ± 0.6	12.4 ± 1.7	15.1 ± 1.7
18:3	0.7 ± 0.2	0.6 ± 0.1	1.5 ± 0.1	1.0 ± 0.1	2.6 ± 0.3	2.6 ± 0.3
20:4	Trace	Trace	0.7 ± 0.1	0.9 ± 0.2	1.4 ± 0.3[d]	2.5 ± 0.4

[a]Means of four to five rats per group and SE.

[b]Differences between CCl_4 treated and control rats are significant at b $p < 0.01$, c $0.02 > p > 0.01$ and d $0.05 > p > 0.02$.

[c]Differences between CCl_4 treated and control rats are significant at b $p < 0.01$, c $0.02 > p > 0.01$ and d $0.05 > p > 0.02$.

[d]Differences between CCl_4 treated and control rats are significant at b $p < 0.01$, c $0.02 > p > 0.01$ and d $0.05 > p > 0.02$.

After a dose of carbon tetrachloride, the structure of rat hepatic triglyceride is specifically altered (1), the percentage of palmitic acid increases and that of linoleic acid decreases at the 1,3-positions in the molecule. It is not clear, however, whether one or both acids in these positions are influenced by CCl_4. The present study was undertaken in order to add more precise information on the effects of CCl_4 on the fatty acid distribution in the different positions as well as on the molecular species of hepatic triglycerides. These studies seem to be of use in clarifying the modification caused by CCl_4 in the hepatic enzymatic systems capable of synthesizing triglyceride (2).

Female Wistar rats, 190-220 g, fed on a commercial pellet ration (Oriental Rat Chow NMF) were fasted for 15 hr and were then given 0.25 ml CCl_4/100 g body weight, as 1:1 (v/v) mixture with liquid paraffin, by stomach tube under light ether anesthesia. Control rats were given liquid paraffin in a similar manner. Rats

TABLE II

Fatty Acid Composition of the Polyunsaturated Species of Hepatic Triglycerides[a]

	Fatty acids, wt %							
	16:0	16:1	18:0	18:1	18:2	18:3	20:4	20:5
6 hr								
CCl_4	30.9	1.9	3.4	14.4	29.5	7.3	10.8	1.8
Control	25.8	2.5	2.9	14.9	33.5	6.3	10.8	3.3
20 hr								
CCl_4	27.2	2.1	1.9	16.2	31.4	7.9	11.4	1.9
Control	25.6	1.3	2.2	15.3	37.0	4.8	11.0	2.8

[a]Values are means of two independent experiments. Pooled liver from five rats per group.

were killed by decapitation and liver lipids were extracted (3). Triglycerides were isolated by thin layer chromatography (TLC) (1,4). The positional distribution of fatty acids on the triglyceride molecule was determined by the combination of pancreatic lipase hydrolysis and the selective phosphorylation of the resulting 1,2-diglycerides by microbial diglyceride kinase (5). Separation of molecular species of triglycerides was carried out by argentation TLC using 0.8-3.0% methanol in chloroform as developing solvent (6). The extraction of the species from the gel was according to Slakey and Lands (7). Pentadecanoic acid was used as the calibration standard. Comparison of the fatty acid compositions between unfractionated and fractionated triglycerides showed that the recovery was within the range of experimental error (± 4%). The fatty acid composition was determined as reported previously (1,8).

Table I shows that hepatic triglyceride from both groups of rats contained palmitic acid mainly at the 1 position and oleic and linoleic acids at the 2 and 3 positions. Although these distribution patterns for major fatty acids were in general comparable with those of Slakey and Lands (7), it is suggested that fasting would influence not only the composition (9), but also the structure of hepatic triglyceride. Triglyceride structure was altered by CCl_4. Thus, after 6 hr, triglyceride from CCl_4 treated rats contained, in comparison to that of the control, more palmitic acid at the 1 position and less oleic acid which increased in the 2 position. Linoleic acid was decreased at the 3-position with a concomitant increase in oleic acid. After 20 hr, the slight decrease in linoleic acid in the 2-position was the sole effect of CCl_4 on the major fatty acids. The effect of CCl_4 was also seen in the minor constituents 6 and 20 hr after administration. These positional changes and time differences in response to CCl_4 agree with those shown in hepatic triglyceride as previously described (1).

Molecular species data for hepatic triglycerides shown in Figure 1 illustrate that CCl_4 treatment generally causes increases in the less unsaturated types of triglyceride species (degree of total unsaturation $\leqslant 3$) and decreases in the more unsaturated species (degree of total unsaturation $\geqslant 4$). Though these changes were more remarkable with 20 hr specimens than with 6 hr specimens, the alteration of the fatty acid composition of intact triglyceride was, in agreement with the previous report (1), more predominant in 6 hr specimens. The species containing 20:4 and 20:5 acids showed the largest decrease, while S_2M, SM_2 and S_2D showed the most prominent increase after CCl_4. The majority (90-95%) of the S_2M, SM_2, S_2D and SD_2 fractions was shown to be constructed with the 2-unsaturated types, while 60-70% of the SMD and M_2D fractions was composed of the 2-diunsaturated types and almost all of the remainder of the former was the 2-monounsaturated type. The changes due to CCl_4 were confined mainly to these major isomers.

The liver slices from rats treated with CCl_4 incorporated more 1-^{14}C-palmitate and oleate into the triglyceride species of S_2M and SM_2 compared to those observed in the controls, while the incorporation of these labeled acyl molecules as well as linoleate into the polyunsaturated species was markedly reduced (Cho et al., unpublished data).

Further attempts to separate the triglyceride family containing 20:4 acid from that containing 20:5 acid by increasing the polarity of the developing solvent for TLC (up to 10% methanol in chloroform) were unsuccessful. The fatty acid composition of the species containing 20:4 and 20:5 acids is, therefore, presented in Table II. In agreement with the previous observation on the composition of whole triglyceride (1), CCl_4 treatment resulted in the decrease in percentage of linoleic acid and the increase in palmitic acid of this fraction, the latter change being remarkable in 6 hr specimens.

These data indicate that CCl_4 influences the overall processes of the triglyceride synthesis from α-glycerophosphate and acyl CoA and the hepatic pool(s) of different triglyceride species. In addition, since the rate of synthesis (10,11), the concentration and fatty acid composition (8) of hepatic glycerophosphatides are specifically modified by CCl_4, it seems likely that the type and size of the hepatic diglyceride pools available for glyceride synthesis are substantially disturbed as previously pointed out (1,3,8). These alterations appear to be induced by the peroxidative degradation of hepatic organells involved in the glyceride metabolism (2).

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Gas Chromatographic Separation of Linoleic Acid Hydroperoxides as Trimethylsilyl Ethers of Methyl Hydroxystearates

ABSTRACT

The primary products, 9- and 13-hydroperoxy-octadecadienoic acids, from lipoxygenase catalyzed oxidation of linoleic acid, were converted into the trimethylsilyl ethers of methyl 9- and 13-hydroxystearates which were completely separated by gas chromatography on an OV-17 methyl silicone (50% phenyl groups), capillary column.

During the oxidation of linoleic acid, catalyzed by lipoxygenase, isomeric 13-hydroperoxy-9-*cis*, 11-*trans*-octadecadienoic acid and 9-hydroperoxy-10-*trans*, 12-*cis*-octadecadienoic acid are formed. Most workers convert these products into more stable compounds, often saturated methyl hydroxy- or ketoacid esters, before subsequent separation and identification work.

Methyl 9- and 13-hydroxystearates have been separated on a silica gel column (1) or by thin layer chromatography (TLC) (2). The column method needs more material than the TLC method which separated most of the methyl 2- to 18-hydroxystearates but had less resolution in the range of the 11- to 14-hydroxy isomers. By combined gas chromatography-mass spectrometry, the methyl 9- and 13-hydroxystearates were identified from their mixed mass spectra though no separation had occurred in the gas chromatography step (3). This method, however, has not been found suitable for quantitative work or routine analysis.

Trimethylsilyl (TMS) ethers of methyl hydroxy acids were easily prepared in good yields (6) and it was also possible to partially separate the TMS-ethers of methyl 12-hydroxystearate and methyl ricinoleate by gas chromatography (4,6). However, no reports have been found about the separation of the TMS-ethers of methyl 9- to 14-hydroxystearates which can be used in the analysis of hydroperoxides from oxidized fatty acids.

The main purpose of the present investigation was to enable a gas chromatographic separation of particularly the TMS-ethers of methyl 9- and 13-hydroxystearates because of the underlying need to analyze the hydroperoxides produced in linoleic acid oxidation. By analyzing a mixture of the 9- and 13-TMS derivatives in a combined gas chromatograph-mass spectrometer with different packed columns, both of the isomers appeared in a single peak as recorded by the ion current detector. Mass spectra were run several times during the elution, and by plotting the intensity of specific mass numbers against retention time, two overlapping peaks were obtained. In this way it was possible to study the tendency for the two isomers to separate on columns of different polarity. The stationary phase that gave the best results was used in a capillary column which separated the isomers completely.

Reference mass spectra were made with methyl 9-, 10-, 11-, 12-, 13- and 14-hydroxystearates. Mixed hydroperoxides obtained by oxidation of linoleic acid with soybean lipoxygenase were converted into the corresponding methyl hydroxystearates (5) which were identified as the 9- and 13-hydroxyacid isomers

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Methyl 9- and 13-hydroxystearates have been separated on a silica gel column (1) or by thin layer chromatography (TLC) (2). The column method needs more material than the TLC method which separated most of the methyl 2- to 18-hydroxystearates but had less resolution in the range of the 11- to 14-hydroxy isomers. By combined gas chromatography-mass spectrometry, the methyl 9- and 13-hydroxystearates were identified from their mixed mass spectra though no separation had occurred in the gas chromatography step (3). This method, however, has not been found suitable for quantitative work or routine analysis.

Trimethylsilyl (TMS) ethers of methyl hydroxy acids were easily prepared in good yields (6) and it was also possible to partially separate the TMS-ethers of methyl 12-hydroxystearate and methyl ricinoleate by gas chromatography (4,6). However, no reports have been found about the separation of the TMS-ethers of methyl 9- to 14-hydroxystearates which can be used in the analysis of hydroperoxides from oxidized fatty acids.

The main purpose of the present investigation was to enable a gas chromatographic separation of particularly the TMS-ethers of methyl 9- and 13-hydroxystearates because of the underlying need to analyze the hydroperoxides produced in linoleic acid oxidation. By analyzing a mixture of the 9- and 13-TMS derivatives in a combined gas chromatograph-mass spectrometer with different packed columns, both of the isomers appeared in a single peak as recorded by the ion current detector. Mass spectra were run several times during the elution, and by plotting the intensity of specific mass numbers against retention time, two overlapping peaks were obtained. In this way it was possible to study the tendency for the two isomers to separate on columns of different polarity. The stationary phase that gave the best results was used in a capillary column which separated the isomers completely.

Reference mass spectra were made with methyl 9-, 10-, 11-, 12-, 13- and 14-hydroxystearates. Mixed hydroperoxides obtained by oxidation of linoleic acid with soybean lipoxygenase were converted into the corresponding methyl hydroxystearates (5) which were identified as the 9- and 13-hydroxyacid isomers

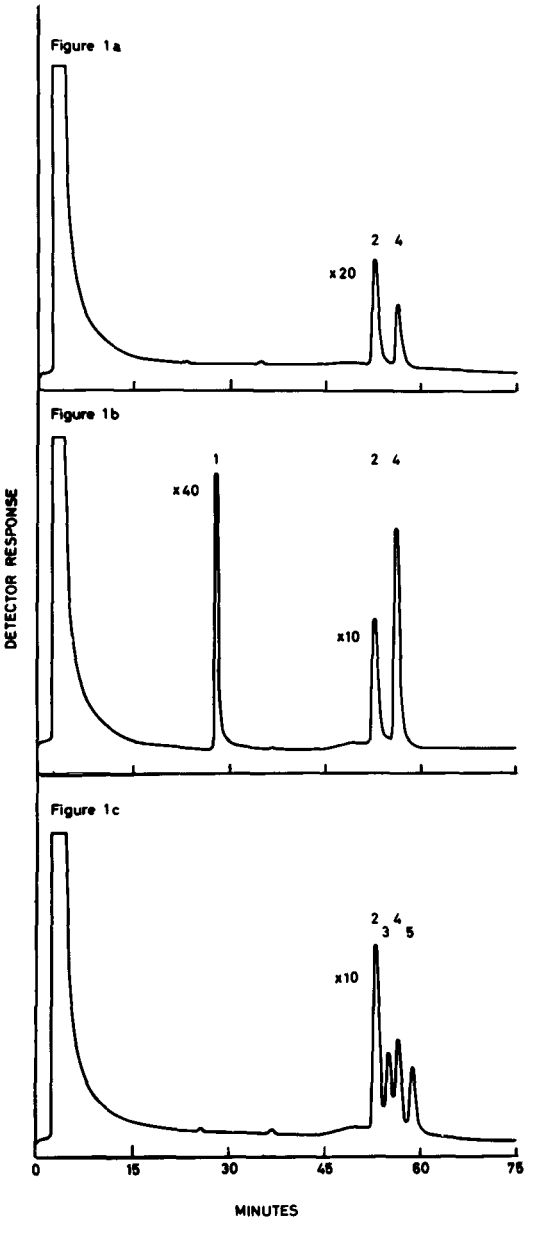

FIG. 1. Gas chromatograms of TMS-ethers of methyl hydroxystearates: a and c; reference compounds; b; compounds derived from linoleic acid hydroperoxides. Peak 1, Methyl stearate. 2, TMS-ether of methyl-9-hydroxystearate. 3, TMS-ether of methyl-12-hydroxystearate. 4, TMS-ether of methyl-13-hydroxystearate. 5, TMS-ether of methyl-14-hydroxystearate.

in an LKB 9000 coupled gas chromatograph-mass spectrometer (3). By this procedure the remaining linoleic acid was converted into methyl stearate which separated from the mixed methyl hydroxystearates on the gas chromatographic column. Both the reference methyl hydroxystearates and those obtained from the enzymatically produced hydroperoxides were converted into their TMS ethers (6). The resulting pyridine solutions, after centrifugation, were analyzed on a 67 m x 0.5 mm i.d. capillary column with a 0.8 μ film of OV-17, methyl silicone (50% phenyl groups), as the liquid phase in a Perkin-Elmer 900 gas chromatograph provided with a flame ionization detector. The inlet split ratio was 16:1, the gas flow through the column 9.5 ml He/min at room temperature; the temperature was 200 C, the injector temperature 270 C.

The gas chromatograms (Fig. 1a,b) of the TMS ethers of methyl 9- and 13-hydroxystearates and of the hydroxy acid isomers derived from the hydroperoxides produced during the oxidation of linoleic acid show that the 9- and 13-isomers were completely separated on the capillary column. In addition, they separated well from the methyl stearate which originated from the excess of linoleic acid in the enzyme incubation medium (Fig. 1b).

We also wanted to know if the column used in the above experiment was able to separate other trimethylsilylated isomers of methyl hydroxystearates within the 9- and 14-isomer range. From the gas chromatogram shown in Figure 1c it is obvious that the 9-, 12-, 13- and 14-TMS substituted isomers could be sufficiently separated for identification and quantitative analysis. Mixed 9- and 10-TMS and 9- and 11-TMS derivatives appeared as a shouldered peak and a double peak respectively (not shown). The absence of peaks around the separated isomers in Figure 1b then indicates that no significant amounts of hydroperoxides other than the 9- and 13-hydroperoxide isomers were produced in the enzymatic oxidation of linoleic acid and therefore the analysis by the present method confirms earlier findings (3,5,7).

The gas chromatographic procedure described above to separate the 9- and 13-TMS compounds has become a valuable tool in the analysis of hydroperoxides produced from unsaturated fatty acids. Unfortunately, other authors (3,5) have not commented on the overall yield of methyl hydroxystearates from hydroperoxides. Therefore, our work now in progress on the mass spectra of the TMS-ethers of methyl 8- to 14-hydroxystearates and a better separation of the 8-, 9-, 10- and 11-TMS isomers also includes quantitative analysis.

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The methyl 9-, 10-, 11-, 12-, 13- and 14-hydroxystearates were donated by E. Stenhagen and L. Morris.

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LETTER TO THE EDITOR

Reactions of Biological Antioxidants: III. Composition of Biological Membranes

Sir. It has been proposed that vitamin E and some part of the coenzymes Q (CoQ) may function together as biological antioxidants (Mellors and Tappel, J. Biol. Chem. 241:4353-4356, 1966). The quantitative levels of the reactive compounds in such function still remain a question in order to judge the validity of the proposal. The question of whether or not the quantities of vitamin E and CoQ in biological membranes relative to peroxidizable unsaturated lipids are of reasonable magnitude to suggest that they may act as effective antioxidants can be answered by considering the lipid composition of the largely membranous mitochondrion. In highly active biological cells, a significant part of the vitamin E and most of the CoQ are found in mitochondria.

We wish to report the results of the calculation of ratios of the unsaturated fatty acids in mitochondria to the total α-tocopherol (α-T) and CoQ, which have been reported for mitochondria. Only data reported for the analyses of rat liver mitochondria and beef heart electron transport particle could be found that included fatty acid composition, CoQ, and α-T determinations based on a common denominator, i.e., weight per cent dry protein. Rat liver mitochondria can contain 384 μmoles fatty acid esters per gram of dry tissue fraction, which contains 63 wt % protein (Getz and Bartley, Biochem. J. 78:307-312, 1961; Getz et al., Biochem. J. 83:181-191, 1962). Of the total mitochondrial fatty acid esters, 49.6 mole % are polyunsaturated fatty acids (PUFA) (Getz, et al., Biochem. J. 83:181-191, 1962). There were 0.66 μmole CoQ per gram protein in a rat liver mitochondrial fraction (Lester and Crane, J. Biol. Chem. 234:2169-2175, 1959). The electron transport particle of beef heart contained 4.2 mg CoQ per gram protein and 0.45 mg α-T per gram protein (Crane, et al., Biochim. Biophys. Acta 31:476-489, 1959).

To calculate mole % CoQ from weight per cent, we chose to consider CoQ_{10} (mol wt 863), which represents the least effect on the results by giving a lower mole ratio of PUFA to biological antioxidant, rather than CoQ_6 or others. In the absence of definitive in vivo studies, we also chose to consider that one tenth of the CoQ might function as an antioxidant. This, in effect, assigns 90% of the function of CoQ to the tasks in electron transport and oxidative phosphorylation, etc., while also minimizing the involvement of CoQ in antioxidant function. It is important too to point out that there are molar excesses of endogenous CoQ over individual cytochromes (Redfearn, Vit. Hormones 24:465-488, 1966), indicating that there could be functions other than those now commonly associated with CoQ.

Calculations from the above values show that the mitochondria may contain both 4.87 μmoles CoQ_{10} per gram protein and 1.04 μmoles α-T per gram protein. Thus, there can be a molar ratio of CoQ_{10} to α-T of 4.7 to 1.0 in the mitochondria. Based, then, on the 0.66 μmole CoQ per gram, there may be 0.14 μmole α-T per gram protein. From the latter value and a calculated 302 μmole PUFA per gram protein, the mitochondrial membrane lipids may have ca. 2100 molecules of PUFA moieties for each molecule of α-T. Similarly, ca. 4600 molecules of PUFA for each CoQ molecule is calculated from 0.066 μmole CoQ per gram for antioxidant function. The combined antioxidant capacity of α-T and CoQ in this model may represent levels of about 0.07 mole % or about 0.1 wt % of the peroxidizable PUFA in mitochondrial membrane lipids.

These calculated levels of biological antioxidants in the membrane are consistent with levels for antioxidant activity in many other systems (Scott, G., "Atmospheric Oxidation and Antioxidants," Elsevier Publishing Co., New York, 1965, p. 282). For example, 0.01 wt % α-T inhibits in vitro oxidation of menhaden oil (Olcott and Einsett, JAOCS 35:159-160, 1958). We conclude from the calculations that biological antioxidants are normally present at adequate levels relative to unsaturated lipids to protect them in membranes from becoming peroxidized significantly in vivo.

It is extremely interesting that Bieri and Poukka (J. Nutr. 100:557-564, 1970) found in rat erythrocytes 1100 molecules of polyunsaturated fatty acids for each molecule of α-T, which was involved in preventing 10% hemolysis in vitro, in cases where the lipids were controlled by diets. The ratios calculated here agree well with these experimental findings.

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Dehydrogenation and Dealkylation of Various Sterols by *Tetrahymena pyriformis*[1]

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ABSTRACT

Sterols are not found in *Tetrahymena pyriformis* when this protozoan is grown in a medium free from exogenous sterols; instead, the principal solid alcohol that can be isolated from the organism is tetrahymanol, a pentacyclic triterpenoid alcohol with an unusual structure. The biosynthesis of tetrahymanol has been shown by appropriate labeling studies to involve a direct, nonoxidative, proton-initiated cyclization of squalene rather than the more commonly found type of mechanism involving squalene 2,3-oxide as an intermediate. In contrast, when *T. pyriformis* is incubated with any one of a wide variety of added sterols, the biosynthesis of tetrahymanol is inhibited and the added sterol is accumulated by the organism and, in most cases, is converted metabolically into one or more other sterols. Four different types of transformation have been observed: the introduction of Δ^5, Δ^7 and Δ^{22} double bonds, and the removal of ethyl groups, but not methyl groups, from C-24.

INTRODUCTION

Tetrahymanol is the name we have given to the pentacyclic triterpenoid alcohol that is the principal component of the nonsaponifiable fraction obtained from *Tetrahymena pyriformis* after this ciliated protozoan has been incubated in a peptone-based medium that is free of exogenous sterols (1). There are also a number of minor nonsaponifiable components, including squalene, ubiquinone and diplopterol (an isomer of tetrahymanol) (2-4). Tetrahymanol was established to have the structure shown in Figure 1 by both physical measurements (1,5) and chemical synthesis (6). This unusual alcohol has recently been found in various other tetrahymenid species (Holz, G.G., Jr., personal communication), and has been shown (7) to be one of the minor constituents isolated (8) from the fern *Oleandra wallichii*.

The biosynthesis of tetrahymanol was postulated (6) to involve a direct, nonoxidative cyclization of squalene with incorporation of the elements of water as indicated in Figure 2, in contrast to the oxidative pathway involved in the biosynthesis of sterols, in which it has been demonstrated that squalene is converted to squalene 2,3-oxide prior to cyclization (9,10). The nonoxidative mechanism illustrated in Figure 2 has been confirmed for *T. pyriformis* by a variety of experiments, both in vivo and in vitro (11-14).

T. pyriformis apparently lacks the capacity for de novo biosynthesis of sterols, as judged by the results of experiments in which the cells were incubated with added ^{14}C-squalene (2,3). Thus, autoradiographic analysis of thin layer chromatograms of the nonsaponifiable material isolated from the cells in these experiments revealed only ^{14}C-tetrahymanol and no ^{14}C-sterols.

When *T. pyriformis* is incubated in a culture fluid containing an added sterol, there are two major consequences of interest: the biosynthesis of tetrahymanol is inhibited and the sterol is accumulated by the cells and, in most cases, undergoes chemical transformations. As described in the following section, we have investigated the scope of both aspects of this mutual interaction between the protozoan and the added sterol, concentrating most of our efforts on determining the chemical fate of the sterol.

RESULTS AND DISCUSSION

Inhibition of Tetrahymanol Biosynthesis

In a standard experiment (2,3), 500 ml of a peptone-based culture fluid (15) containing various amounts of added sterol was inoculated with an axenic culture of *T. pyriformis* (about 1 x 10^6 cells) and the incubation was allowed to proceed for 36-40 hr at 28 C. The number of cells was then estimated with a Coulter particle counter; a typical value was 4 x 10^8 cells. The cells were harvested (16) and the tetrahymanol was isolated in the usual way. The amount of tetrahymanol was estimated by quantitative gas liquid chromatography (GLC); an F & M Model 400 gas chromatograph equipped with an SE-52 column, a flame ionization detector, and a disc

[1]One of 12 papers to be published from the "Sterol Symposium" presented at the AOCS Meeting, New Orleans, April 1970.

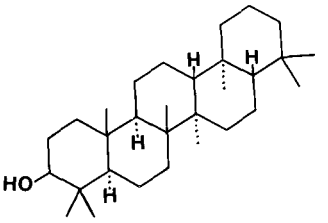

FIG. 1. Tetrahymanol.

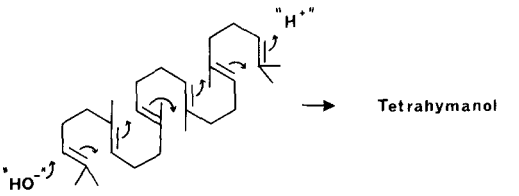

FIG. 2. Schematic illustration of the enzymatic cyclization of squalene in *T. pyriformis*.

integrator was used for these analyses. The extent of inhibition of tetrahymanol biosynthesis varied with the sterol content of the culture fluid. For the particular case of added cholesterol, the results (2,3) are given in Table I; since the amount of tetrahymanol detected in the experiments with 5.0 and 10.0 mg of added cholesterol corresponds roughly to the amount present in the initial inoculum, it is evident that the inhibition of tetrahymanol biosynthesis under these conditions is essentially complete.

Similar inhibition has been found for all of the numerous other C_{27}, C_{28} and C_{29} sterols that we have tested (R.L. Conner, J.R. Landrey and F.B. Mallory, unpublished results), including cholesta-5,7,22-trien-3β-ol and ergosterol (see below). On the other hand, tetrahymanol biosynthesis was not inhibited by tetrahymanol itself (Ibid.), nor was it inhibited by lanosterol or 24,25-dihydrolanosterol (Ibid.), two other C_{30} compounds having a gem-dimethyl group at C-4.

Metabolic Transformations of Sterols

We have incubated *T. pyriformis* with a variety of different sterols under the conditions outlined in the previous section, and we have isolated and characterized as many of the transformation products as possible. In most cases a mixture of sterols was obtained, which was separated chromatographically by methods described earlier (17,18) for the particular case of cholesterol as the added sterol. The techniques (17,18) used to identify the individual sterols included measurements of UV, IR, NMR, and mass spectra, GLC retention times, and Liebermann-Burchard responses. In most cases, authentic samples of the sterols were available for comparison. We also developed a method for determining the presence or absence of a Δ^{22} double bond by periodate-permanganate cleavage of such double bonds to give low molecular weight carboxylic acids (e.g., isovaleric acid from a Δ^{22} sterol in the C_{27} series) that are detected quantitatively by GLC (19).

TABLE I

Tetrahymanol Content of *T. pyriformis* Incubated With Various Concentrations of Added Cholesterol

Cholesterol added to culture, mg/500 ml	Tetrahymanol formed, μg/10^8 cells)	Number of cultures assayed
None	880 ± 88[a]	16
2.5	320 ± 66	5
5.0	12 ± 12	6
10.0	8 ± 6	3

[a]Standard deviation.

The results of these studies indicate that *T. pyriformis* can introduce Δ^5, Δ^7 and Δ^{22} double bonds into sterols, and also can remove the C-24 ethyl group present in C_{29} sterols. The individual studies that form the basis for these generalizations are described below.

The first studies we undertook involved cholesterol as the added sterol (17,18). From the mixture of sterols present in the nonsaponifiable fraction obtained from cholesterol-incubated cells we obtained cholesta-5,7-dien-3β-ol, cholesta-5,22-dien-3β-ol, cholesta-5,7,22-trien-3β-ol, and some recovered cholesterol. These results suggest that cholesterol is converted to cholesta-5,7,22-trien-3β-ol by two independent pathways as indicated in Figure 3. This suggestion has been confirmed by our observation (Conner et al., unpublished results) that incubations of *T. pyriformis* either with cholesta-5,7-dien-3β-ol or with cholesta-5,22-dien-3β-ol result in the efficient transformation of the added dienol to the trienol, cholesta-5,7,22-trien-3β-ol.

Incubations with cholesta-5,7,22-trien-3β-ol as the added sterol led to the recovery of this trienol as the only detectable sterol in the nonsaponifiable fraction obtained from the cells (Ibid.).

We have extended these studies to two other C_{27} sterols, 5α-cholest-7-en-3β-ol (Δ^7) and 5α-cholestan-3β-ol (Δ^0). In each case, incubation with *T. pyriformis* resulted in the formation of cholesta-5,7,22-trien-3β-ol ($\Delta^{5,7,22}$) together with other less extensively dehydrogenated sterols (Ibid.) Thus, both cholesta-5,7-

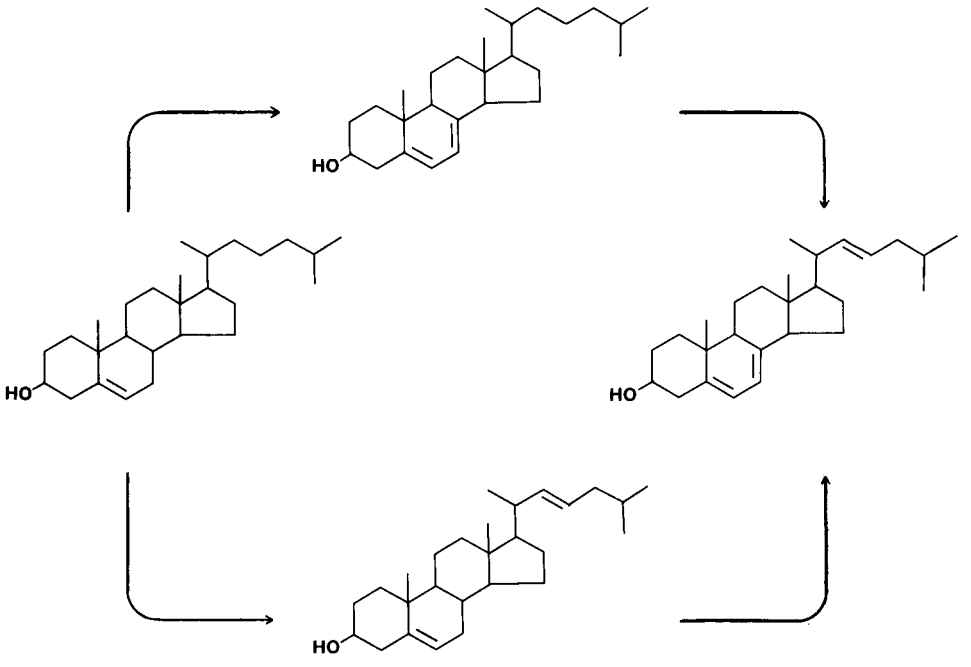

FIG. 3. Two pathways for the conversion by *T. pyriformis* of cholesterol to cholesta-5,7,22-trien-3β-ol, proceeding by way of either cholesta-5,7-dien-3β-ol or cholesta-5,22-dien-3β-ol.

dien-3β-ol ($\Delta^{5,7}$) and 5α-cholesta-7,22-dien-3β-ol ($\Delta^{7,22}$) were detected as products from incubations of 5α-cholest-7-en-3β-ol; and 5α-cholest-22-en-3β-ol (Δ^{22}), 5α-cholesta-7,22-dien-3β-ol ($\Delta^{7,22}$), and (probably) cholesta-5,22-dien-3β-ol ($\Delta^{5,22}$) were all detected as products from incubation of 5α-cholestan-3β-ol. On the basis of the observation that 5α-cholest-7-en-3β-ol is converted in part to cholesta-5,7-dien-3β-ol ($\Delta^{7} \rightarrow \Delta^{5,7}$), and the tentative conclusion that 5α-cholestan-3β-ol is converted in part to cholesta-5,22-dien-3β-ol ($\Delta^{0} \rightarrow \Delta^{5,22}$), it follows that *T. pyriformis* can introduce a Δ^5 double bond regardless of whether or not a Δ^7 double bond is already present. Similarly, the observed $\Delta^5 \rightarrow \Delta^{5,7}$ and $\Delta^0 \rightarrow \Delta^{7,22}$ conversions show that this protozoan can introduce a Δ^7 double bond with or without a Δ^5 double bond being present.

The scheme given in Figure 4 illustrates the 12 conceivable dehydrogenation steps connecting the eight C_{27} sterols under consideration. Our studies to date explicitly establish the existence of seven of these steps (indicated by full-line arrows), and allow plausibility arguments to be made concerning the existence of the remaining five steps (indicated by dashed-line arrows). For example, it can be argued that there is no reason to doubt that an incubation starting with the $\Delta^{7,22}$ dienol would lead to the formation of the $\Delta^{5,7,22}$ trienol, since the analogous conversion of the Δ^7 to the $\Delta^{5,7}$ sterol has been unambiguously demonstrated to occur.

None of the incubations described above resulted in the formation of detectable amounts

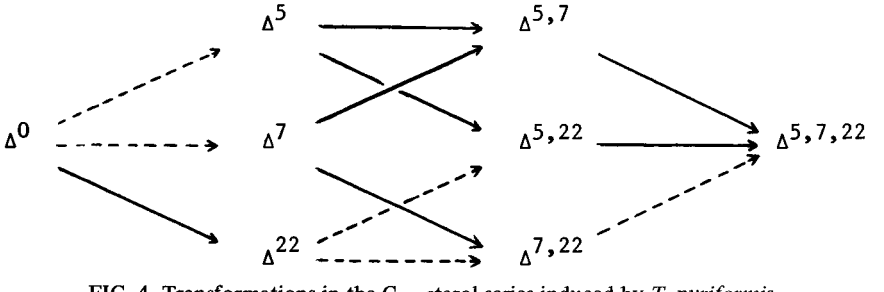

FIG. 4. Transformations in the C_{27} sterol series induced by *T. pyriformis*.

of sterols in which a double bond originally present had become saturated.

The stereochemistry of the introduction of the Δ^7 and Δ^{22} double bonds in the conversion of cholesterol to cholesta-5,7,22-trien-3β-ol by *T. pyriformis* has been established by recent studies in other laboratories (20,21). Thus, the overall transformation involves the loss of the 7β-, 8β-, 22-*pro-R*-, and 23-*pro-S*-hydrogens.

Our discovery of the capacity of *T. pyriformis* to introduce Δ^{22} double bonds has proved to be useful in the solution of other problems in sterol biochemistry. For example, the stereochemical relationship between the C-2 hydrogens in mevalonic acid and the C-22 hydrogens in cholesterol derived from mevalonic acid in rat liver remained unverified until a recent study was carried out (20) in which cholesterol derived in this way from either (2*R*)- or (2*S*)-[2-^{3}H; 2-^{14}C]-mevalonic acid was subjected to stereospecific (20,21) dehydrogenation by *T. pyriformis* to give cholesta-5,7,22-trien-3β-ol; the results of this study (20) indicate that the 22-*pro-R*-hydrogen of cholesterol is derived from the 2-*pro-R*-hydrogen of mevalonic acid, and that the 22-*pro-S*-hydrogen is derived from the 2-*pro-S*-hydrogen. The introduction of a Δ^{22} double bond by *T. pyriformis* followed by oxidative cleavage of this double bond (e.g., by periodate-permanganate or by ozone) has been used by two groups as a degradation scheme that allows the position of tritium to be determined in various tritium-labeled side chains. For example, this approach was used (22) to provide strong support for the suggestion that the mechanism of the reduction of the Δ^{24} double bond in the conversion of desmosterol (cholesta-5,24-dien-3β-ol) to cholesterol involves electrophilic addition of an enzyme-bound proton to C-24 followed by nucleophilic delivery of hydride to C-25 from the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH). In another study (23), the biosynthetic origin of the C-20 hydrogen of cholesterol derived from mevalonic acid in a rat-liver system was demonstrated by the observation that cholesterol biosynthesized from (4*R*)-[4-^{3}H; 2-^{14}C]-mevalonic acid contains tritium at C-20.

We have also carried out a limited number of incubations with sterols in the C_{28} series (Conner et al., unpublished results), and the results are analogous to those obtained from our studies of C_{27} sterols. Thus, both 24α-methylcholesterol (campesterol) and 24β-methylcholesterol are transformed by *T. pyriformis* into a mixture of the corresponding $\Delta^{5,7}$ dienol and $\Delta^{5,7,22}$ trienol with retention of the C-24 methyl substituent. Brassicasterol (24β-methyl, $\Delta^{5,22}$) is also converted to ergosterol (24β-methyl, $\Delta^{5,7,22}$), and ergosterol itself is recovered unchanged after incubation with the protozoan. In no case was any C_{27} sterol detectable (by GLC) in the material isolated from cells that had been incubated in the presence of a C_{28} sterol.

Incubations of some sterols in the C_{29} series (Ibid.) resulted in the loss of the C-24 ethyl substituent as well as dehydrogenation to give the $\Delta^{5,7,22}$ pattern of double bonds. Thus β-sitosterol (24α-ethyl, Δ^5), stigmasterol (24α-ethyl, $\Delta^{5,22}$), and poriferasterol (24β-ethyl, $\Delta^{5,22}$) were all converted by *T. pyriformis* to a mixture of the corresponding 24-ethyl-$\Delta^{5,7,22}$ trienol together with the dealkylated trienol, cholesta-5,7,22-trien-3β-ol. An incubation starting with a synthetic sample of the 24α-ethyl-$\Delta^{5,7,22}$ trienol (stigmasta-5,7,22-trien-3β-ol) also resulted in the formation of cholesta-5,7,22-trien-3β-ol as the only detectable product.

Thus it appears that *T. pyriformis* removes either 24α- or 24β-ethyl groups from C_{29} sterols, but removes neither 24α- nor 24β-methyl groups from C_{28} sterols.

There are three aspects of our results that we feel are of interest for further study; the mode of action of sterols in the inhibition of tetrahymanol biosynthesis; the mechanism of introduction of Δ^5, Δ^7, and Δ^{22} double bonds; and the mechanism of the de-ethylation at C-24.

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The Biosynthesis of Cholesterol and Other Sterols by Brain Tissue: I. Subcellular Biosynthesis in Vitro[1]

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ABSTRACT

The biosynthesis of cholesterol by subcellular particles from rat brain was studied with several labeled cholesterol precursors as substrates. Rats from two age groups were used for preparation of the subcellular fractions: 15-day-old and adult. Microsomes and a soluble fraction were required for maximum biosynthesis of ^{14}C-nonsaponifiable material. The latter was synthesized in good yield by subcellular fractions from both age groups, but 90% or more was present as ^{14}C-squalene, when either U-^{14}C-glucose, 2-^{14}C-sodium acetate or 2-^{14}C-mevalonic acid was the radioactive substrate. Neither ^{3}H-squalene oxide nor ^{14}C-lanosterol was converted to sterol when incubated with microsomal + soluble preparations, but some 4% of ^{14}C-desmosterol was converted to cholesterol by adult preparations. Thus a metabolic block, largely between squalene to desmosterol, exists in isolated microsomal + soluble preparations from both 15-day-old and adult rat brain.

INTRODUCTION

Some problems concerning brain cholesterol metabolism that have intrigued and challenged our laboratory for a number of years are discussed. One of these is that the incubation of brain tissue preparations with established cholesterol precursors has indicated that as the mammalian organism matures, its brain loses the capacity to synthesize one of its most important lipid constituents, cholesterol (1,2). This concept was challenged several years ago by experiments in which labeled cholesterol precursors such as ^{14}C-acetate and ^{14}C-mevalonic acid were injected intracisternally (3) or intracerebrally (4). On intracerebral injection of such cholesterol precursors in adult rats, it is possible to obtain labeled cholesterol of considerably higher specific activity than that obtained from incubations (5).

Over the past 10 years it seems to have been gradually accepted that the adult brain does have a small but detectable capacity to synthesize cholesterol (6-21). However, this small capacity for synthesis in vitro seems to be at variance with the level of synthesis seen in intracerebral injection. Intracerebral injection experiments do not seem to have been generally accepted as valid (22). Because such an experiment involves a brain wound, it is possible that the biochemical events following this might be related to the wounding itself, with the cholesterol biosynthesis being a secondary phenomenon. Actually there is no known biochemical or histological basis for this assumption. Davison has suggested that the increased biosynthetic uptake of precursors as a result of intracerebral injection could be due to increased access of the tissue to the precursor (23). The results we obtained by injecting cholesterol precursors intracerebrally are a nonartifactural phenomenon of the adult brain tissue itself.

One might ask, why pursue this particular

TABLE I

Incorporation of 2-^{14}C-Mevalonic Acid Into Brain Subcellular Fractions[a]

Fractions	^{14}C-Content	
	Nonsaponifiable, dpm	Digitonin-precipitable dpm
15,000 x *g* supernatant	125,000	1,530
112,000 x *g* supernatant (soluble)	109,000	470
Microsomes + soluble	123,000	1,820
Microsomes only	1,050	0
Mitochondria only	1,060	0

[a]Each incubation contained 0.25 μc 2-^{14}C mevalonic acid and tissue equivalent to 1.0 *g* of wet adult rat brain tissue. Cofactors were as indicated in Table II.

[1]One of 12 papers to be published from the Sterol Symposium presented at the AOCS Meeting, New Orleans, April 1970.

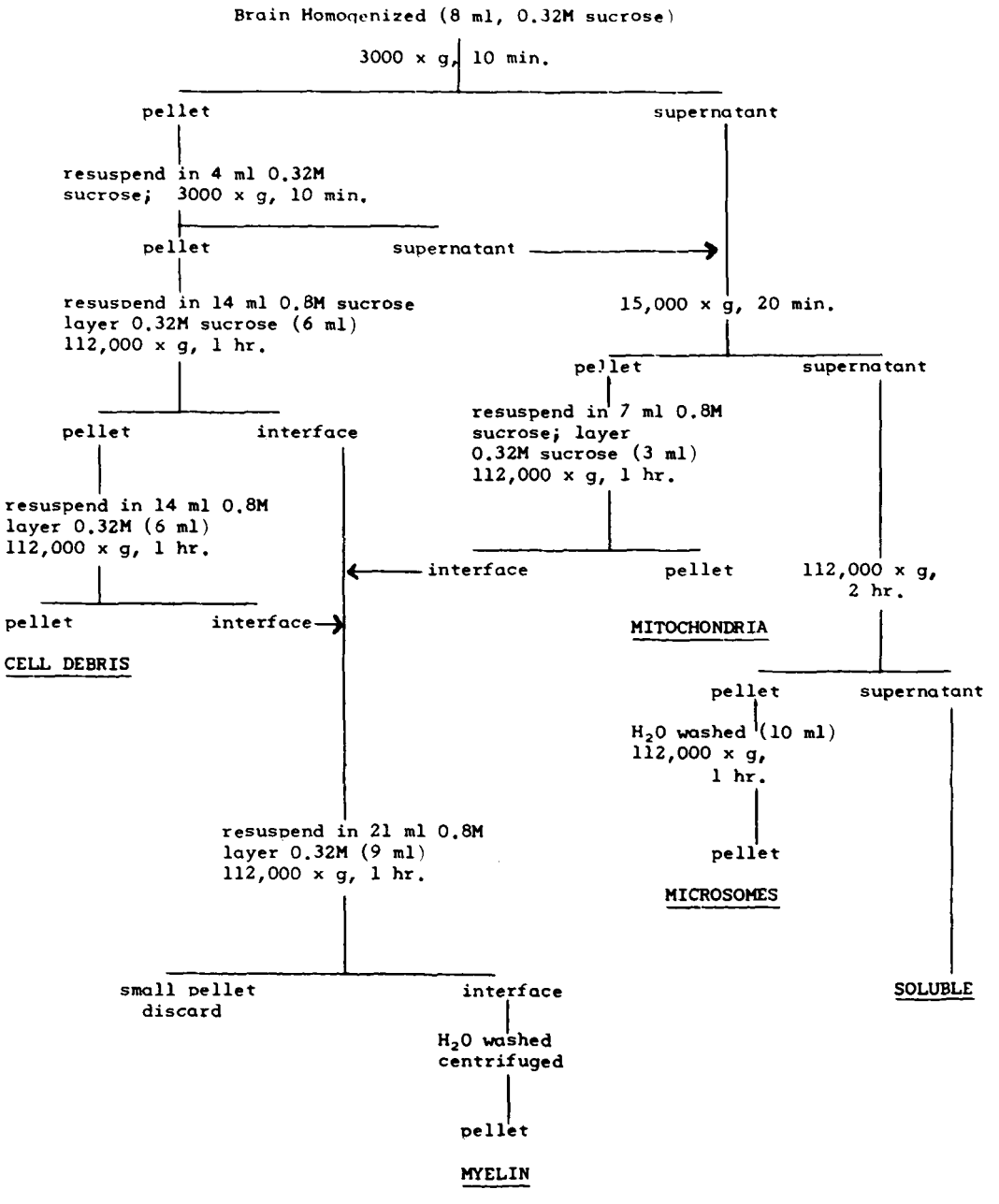

FIG. 1. The general isolation scheme used for the separation of the subcellular fractions of brain tissue.

problem? It is not unusual for a tissue, especially brain tissue, to behave differently in the test tube than it does in vivo. One major reason, we believe, is that since cholesterol represents a single chemical entity constituting some 10% of the dry weight of brain, it must accordingly have a primary and critical biochemical function in the central nervous system. At present this function appears to be connected with cholesterol as an integral part of the myelin sheath. When disintegration of this sheath occurs the effect on the animal is disastrous (24). If for no other reason than to establish the role of cholesterol in the formation and maintenance of the myelin sheath, we should know as much as possible about the

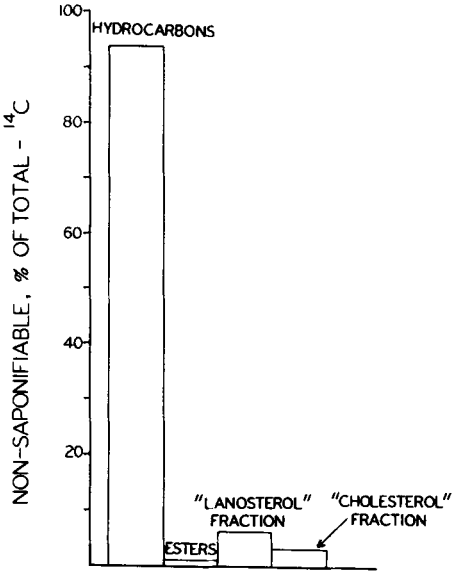

FIG. 2. Distribution of labeled material in the non-saponifiable fraction after alumina column chromatography of adult rat brain microsomal + soluble incubation.

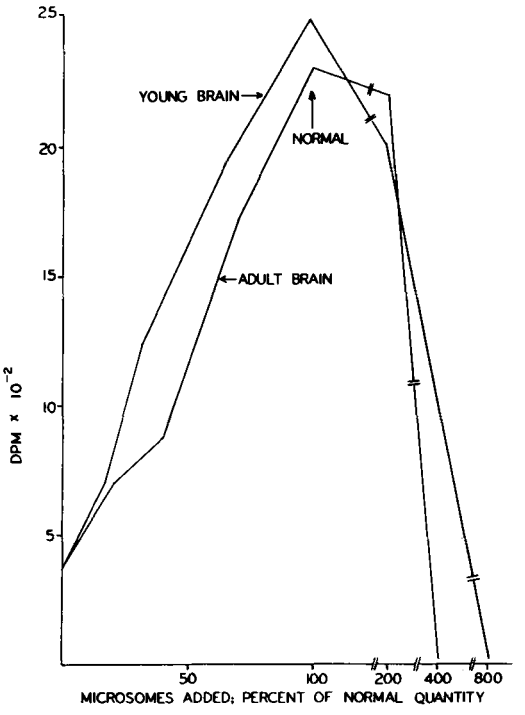

FIG. 3. Effect on amount of digitonin-precipitable material formed by addition of increasing amounts of microsomes from rat brain to a soluble fraction, also from rat brain. Each incubation contained soluble fraction equivalent to 0.5 g of wet tissue and the per cent of the normal equivalent amount of microsomes, as indicated. 0.25 μc of 2-^{14}C-mevalonic acid was used per incubation.

biosynthesis and metabolism of this sterol.

A second reason for pursuing this problem is the question of regeneration. If the adult brain cannot synthesize this important constituent, there seems to be little hope in conducting experiments designed to accomplish regeneration of adult CNS tissue. If the potentiality for regeneration exists, then several possibilities become apparent; localized administration of precursors to affect regeneration or drug-induced regeneration of tissue are two of these possibilities.

Finally, there appear to be an increasing number of studies involving the addition of metabolites to brain homogenates with the intention of influencing brain cholesterol biosynthesis (25,26). The properties of such homogenates should be thoroughly investigated. A study of cholesterol biosynthesis at the subcellular level, complementing the previous work on cell-free preparations (27), therefore is in order.

TABLE II

Optimum Cofactors for Brain Subcellular Biosynthesis of Total Nonsaponifiable and Digitonin-Precipitable Material Using Various Radioactive Substrates[a]

	Substrates		
Cofactor	2-^{14}C-Mevalonic acid (DBED salt), μm	2-^{14}C-Acetic acid (Na Salt), μm	U-^{14}C-Glucose, μm
GSH	8.20	0	0
DPNH	3.50	1.41	4.32
ATP	2.12	5.20	5.20
G-6-P	8.25	3.30	9.90
Mg^{++}	12.3	4.92	14.8

[a]Each incubation contained microsomal and soluble fractions equivalent to 0.5 g of wet brain tissue. The cofactors indicated above are identical for both young and adult preparations except the G-6-P requirement for young preparations containing U-^{14}C-glucose. In this case the G-6-P requirement was one third that indicated for the adult above.

TABLE III

Effect of Individual Cofactors on Nonsaponifiable and Digitonin-Precipitable Material[a]

Added	^{14}C-Content	
	Nonsaponifiable, dpm	Digitonin-precipitable, dpm
Complete	166,000	5,800
No G-6-P	122,000	3,500
No GSH	135,000	1,640
No Mg^{++}	61,000	120
No DPNH	2,400	33
No ATP	1,150	12

[a]Each incubation contained 0.25 μc 2-^{14}C-mevalonic acid and microsomal + soluble fractions from adult rat equivalent to 1.0 g of wet brain tissue. Cofactors were as indicated in Table II.

MATERIALS AND METHODS

Animals

The rats used were purchased from National Laboratory Animal Co., St. Louis. The adult animals were at least six months old and weighed at least 250 g. The young rats were 10-15 days old. They will subsequently be referred to as 15-day-old rats. Animals of both sexes were used.

Preparation of Tissue

The subcellular fractions were prepared in the manner outlined in Figure 1. Each brain was homogenized in a glass homogenizer with a loose-fitting Teflon pestle. All manipulations were carried out at 4 C and no more than eight complete pestle plunges were made. In some instances the soluble fraction was decanted and recentrifuged for an additional 2 hr. However, no biosynthetic difference was found between this soluble fraction and the one prepared in the normal manner. Numerous experiments were also carried out in which the soluble fractions were filtered through a Millipore filter (0.45 μ). The filtered soluble fractions, however, behaved biosynthetically the same as the unfiltered fractions; this step was subsequently deleted.

Incubations

Incubations were performed in 50 ml flasks shaken gently at 37 C for 20 hr unless otherwise indicated. The flasks were covered with aluminum foil. Because of the length of many of the incubations they were carried out with and without antibiotics. No difference was found, as previously indicated (27). Unless otherwise indicated the time of incubation was 20 hr. The volumes of the incubation mixture ranged from 1.5 to 2.5 ml. The cofactors used were in the proportions and amount indicated in Table II. Single determinations were made in the pig brain acetone powder incubations. All other incubations were in duplicate.

Chromatography

Column chromatography was carried out on 60 g alumina columns as previously described (27).

Reverse-phase thin layer chromatography (TLC) for the separation of desmosterol from cholesterol was run on Kieselguhr G impregnated with paraffin oil. The solvent system was acetone-water (4:1), as described by De Souza and Nes (28).

Saponification and Isolation of Lipid Fractions

To the incubations was added 30 ml of water-ethanol (1:1) containing 15% KOH (w/v). The alkaline solution was then immediately extracted with petroleum ether five times. The combined organic phases were washed three

TABLE IV

Incubation of Brain Subcellular Fractions With ^{14}C-Labeled Substrates

Age of animal	Precursor	Nonsaponifiable, dpm	Digitonin-precipitable, dpm
15 Days			
	U-^{14}C-Glucose	58,900	590
	2-^{14}C-Na Acetate	29,900	560
	2-^{14}C-Mevalonic Acid	155,000	600
Adult			
	U-^{14}C-Glucose	64,500	820
	2-^{14}C-Na Acetate	41,300	240
	2-^{14}C-Mevalonic Acid	151,000	900

[a]Each incubation contained, in addition to microsomal and soluble preparations representing 0.5 g wet brain, 2.0 μc U-^{14}C-glucose, 2.0 μc 2-^{14}C-sodium acetate, and 0.25 μc 2-^{14}C-mevalonic acid. Cofactors were as indicated in Table II.

TABLE V

Effect of Incubation Time of Adult and Young Rat Brain Subcellular Fractions (Microsomes + Soluble)

Incubation[a] time, hr	^{14}C-Content			
	Nonsaponifiable, dpm/g wet wt		Digitonin-precipitable, dpm/g wet wt	
	Adult	Young	Adult	Young
0.5	33,700	---	310	---
1.0	75,000	42,300	560	130
2.0	111,000	181,000	940	400
4.0	118,000	198,000	1,970	1,080
6.0	125,000	---	3,300	---
8.0	111,000	192,000	3,280	---
12.0	107,000	194,000	4,130	1,160

[a]Each incubation contained 0.25 μc 2-^{14}C-mevalonic acid and microsomal + soluble fractions from adult or young rat equivalent to 1.0 g of wet brain tissue. Cofactors were as indicated in Table II.

times with 50 ml of water and then dried under N_2. Digitonides were prepared from the nonsaponifiable fractions by the Sperry-Webb method (29). Free sterol was obtained from the digitonides by the method of Frame (30). In certain instances these sterols were converted to the dibromides and then debrominated by the method of Schwenk and Werthessen (31). This method, however, does not separate desmosterol from cholesterol. Approximately 30% of the former sterol is retained by cholesterol in this method. (This laboratory, unpublished observations).

Radioactivity

Radioactivity was determined using an Ansitron scintillation spectrometer as previously described (27).

Chemicals

All cofactors were obtained from Sigma Chemical Co., St. Louis, Mo., except the $MgCl_2 \cdot 6H_2O$, which was Mallinckrodt A.R. Grade. All other reagents were Mallinckrodt A.R. Grade. The 2-^{14}C-acetate (sp. act. 2.0 mc/m mole), U-^{14}C-glucose (sp. act. 4.92 mc/m mole), 2-^{14}C-mevalonic acid (sp. act. 5.82

TABLE VI

Incorporation of Cholesterol Precursors in Brain Subcellular Fraction Incubations[a]

Age of animal	Subcellular fraction(s)	Precursor	Digitonni-precipitable, dpm	Cholesterol dibromide, dpm
15 Days	Soluble only			
		^{14}C-Squalene	740	0
		^{14}C-Lanosterol	130,000	9
	Microsomal + soluble			
		^{14}C-Squalene	960	0
		^{3}H-Squalene Oxide	2,240	---
		^{14}C-Lanosterol	108,000	23
Adult	Soluble only			
		^{14}C-Squalene	1,130	0
		^{14}C-Lanosterol	35,000	8
	Microsomal + soluble			
		^{14}C-Squalene	1,590	0
		^{3}H-Squalene Oxide	1,560	---
		^{14}C-Lanosterol	143,000	0

[a]Each incubation contained subcellular fraction(s) equivalent to 1 g of wet brain. Labeled materials being incubated were suspended in a drop of Tween 80. Amounts of radioactive materials added per incubation: ^{14}C-squalene, 0.25 μc; ^{3}H-squalene oxide, 0.086 μc; ^{14}C-lanosterol, 0.16 μc.

TABLE VII

Utilization of 2-^{14}C-Mevalonic Acid by an Acetone Powder of Whole Pig Brain[a]

Acetone powder, mg	^{14}C-Content	
	Nonsaponifiable, dpm	Digitonin-precipitable, dpm
100	95,600	1,820
200	57,800	1,360
500	83,800	650

[a]Each incubation contained 0.25 μc 2-^{14}C-mevalonic acid, 16.3 μm reduced glutathione, 7.00 μm DPNH, 4.25 μm ATP, 16.5 μm glucose-6-phosphate, and 24.6 μm Mg^{++}.

mc/m mole) and 26-^{14}C-desmosterol (sp. act. 45.0 mc/m mole) were purchased from New England Nuclear Corp., Boston, Mass.

^{14}C-Lanosterol was obtained biosynthetically from 2-^{14}C-mevalonic acid as described by Moller and Chen (32). ^{14}C-Squalene was produced by incubation of 2-^{14}C-mevalonic acid with a liver homogenate as previously described (33). The squalene was purified by alumina column chromatography and TLC. The material was found to be pure by radio-gas-liquid chromatography.

Commercial whole pig brain acetone powder was purchased from the Sigma Chemical Co.

RESULTS

Subcellular Fractions Responsible for Sterol Biosynthesis

In Table I are shown the subcellular fractions of adult brain responsible for biosynthesis of ^{14}C-nonsaponifiable material with 2-^{14}C-mevalonic acid as the labeled substrate. As with analogous liver preparations, the microsomal + soluble fractions are clearly responsible for maximum synthesis of digitonin-precipitable material, although the soluble fraction exhibited some activity in this respect by itself.

Composition of the Nonsaponifiable Fraction Synthesized by Microsomal + Soluble Preparations

The distribution of counts in a nonsaponifiable fraction biosynthesized by an adult rat brain microsomal + soluble preparation, with 2-^{14}C-mevalonic acid as the substrate, is shown in Figure 2. This distribution was obtained by fractionation of nonsaponifiable material on an aluminum column. It will be noted that approximately 95% of the counts are present in the hydrocarbon (squalene). A small amount was present in the "ester" region, somewhat more in the "lanosterol" (4,4-dimethyl) region, and approximately 3% in the sterol (cholesterol) region. The profile of 15-day-old brain incubations under the same conditions showed no significant differences from the adult brain distribution.

Cofactor Requirements

Since it has been found that the type and quantity of cofactors present in the incubation medium influences considerably the nature of the nonsaponifiable in adult brain cell-free extracts (27), this problem was investigated for microsomal + soluble fractions from adult and young rat brains. The quantities established as giving "optimum" ^{14}C-labeled digitonin-precipitable material are shown in Table II, where

TABLE VIII

Comparison of Adult Rat Brain Subcellular Fractions to Those of Adult Rat Liver

Subcellular fractions[a]	^{14}C-Content of nonsaponifiable fraction, dpm	^{14}C-Content of digitonin-precipitable material, dpm
Brain soluble only	168,000	800
Brain soluble + liver microsomes	149,000	29,500
Brain soluble + brain microsomes	170,000	2,700
Liver soluble only	204,000	13,000
Liver soluble + liver microsomes	195,000	111,000
Liver soluble + brain microsomes	187,000	22,200

[a]Each incubation contained 0.25 μc 2-^{14}C-mevalonic acid. Each subcellular fraction indicated is equivalent to 1 g of wet brain or liver. Cofactors were added as for brain subcellular fractions indicated in Table II.

2-^{14}C-mevalonic acid, 2-^{14}C-sodium acetate, and U-^{14}C-glucose respectively were utilized as radioactive precursors. With the exception of G-6-P when U-^{14}C-glucose was used as the substrate, the quantities required for optimum biosynthesis of sterol were the same for young and old brain preparations.

While the presence of TPNH is needed for the production of digitonin-precipitable material in liver subcellular fractions, it was found that the addition of TPNH or a TPNH generating system to the brain subcellular incubations had no effect on the production of digitonin-precipitable material. The same has been found previously with brain cell-free preparations.

It was thought that possibly one of the reasons for the low biosynthetic capacity of our subcellular system was the release of potent adenosine triphosphatases in the isolation of the microsomes. However, neither the constant addition of ATP nor the addition of an ATP generating system had any effect on the production of digitonin-precipitable material.

The microsomal + soluble preparations from adult brain exhibited an absolute requirement for ATP and DPNH, both of which had sharp optima. This requirement is illustrated in Table III. Magnesium, GSH and G-6-P were also required to a lesser extent, in that order. The optima of these cofactors were not well defined. The variation of the amounts of cofactors required for different substrates cannot be explained at this time.

Comparison of Young and Adult Microsomal + Soluble Biosynthetic Capacity

Using three different ^{14}C-labeled substrates, a comparison was made of the ability of young and adult microsomal + soluble preparations to biosynthesize ^{14}C-labeled nonsaponifiable material. The amount of the latter precipitated by digitonin was also determined. The results are shown in Table IV. While there were differences in both groups depending upon whether 2-^{14}C-mevalonic acid, U-^{14}C-glucose or 2-^{14}C-acetate was used as the substrate, as a whole there were no markedly different values in comparing young or adult brain preparations with the same labeled substrate, either in terms of total ^{14}C-nonsaponifiable or ^{14}C-digitonin-precipitable fractions.

Rate of Formation of Nonsaponifiable and Digitonin-Precipitable Material

One minor difference was found in the rate at which nonsaponifiable and digitonin-precipitable material was synthesized by young and adult preparations. This is illustrated in Table V. Whereas the young brain preparations reached a maximum biosynthetic capacity in 4 hr, the adult brain preparations required some 10 hr for maximum activity. Previous experiments have shown that after 12 hr there is a very gradual but significant increase in incorporation up to 20 hr. Again, however, it will be noted that in all cases, for both young and adult brain, most of the counts were not precipitated by digitonin, despite the fact that ^{14}C-nonsaponifiable material was formed in good yield. Most of the latter, as illustrated in Figure 2, was squalene which had not been converted to sterol.

Incorporation of Other Labeled Precursors Into Adult Brain Microsomal + Soluble Preparations

At this point of the investigation it appeared that the major block in the mevalonic acid to cholesterol sequence for both young and adult microsomal + soluble preparations was in the squalene to cholesterol steps. To substantiate this, ^{14}C-squalene, ^{3}H-squalene oxide, and ^{14}C-lanosterol respectively were used as substrates. The results are shown in Table VI, and substantiate the results previously illustrated with 2-^{14}C-mevalonic acid, 2-^{14}C-sodium acetate or U-^{14}C-glucose; namely, only a small biosynthetic capacity for sterol formation. When 26-^{14}C-desmosterol was incubated with adult rat brain microsomal + soluble preparations, a 4.4% conversion to cholesterol was found after the two compounds were separated by reverse-phase TLC.

Biosynthesis of ^{14}C-Nonsaponifiable From Pig Brain Acetone Powder

As indicated in Table VII, an exploratory experiment with a commercial whole pig brain acetone powder showed that even this type of preparation can synthesize squalene in reasonably good yield with 2-^{14}C-mevalonic acid as the substrate. The amount of ^{14}C-sterol formed was again in low yield.

The Cause of the Squalene to Cholesterol Biosynthetic Block in Brain Microsomal + Soluble Preparations

Inability to convert squalene to cholesterol could be due to a deficiency of some factor or the presence of an inhibitor in either the microsomal or soluble preparations. The possibility of an inhibitor in the microsomes was indicated by the data in Figure 3. When varying volumes of brain microsomal suspension were added to a fixed volume of soluble fraction the curve shown in Figure 3 was obtained. A linear relationship was exhibited between the amount of ^{14}C-sterol formed and the volume of microsomal suspension in the incubation

medium up to the point where the amounts of the respective fractions were equivalent ("normal") to that in brain tissue. Addition of microsomes above this normal level, for both young and adult preparations, produced a marked inhibition in capacity to produce ^{14}C-labeled sterol, with 2-^{14}C-mevalonic acid as the radioactive substrate.

However, the soluble fraction may be also responsible. In Table VIII data are shown for the incubation of brain soluble and microsomal fractions with their opposite but analogous fractions from liver. It can be seen that neither microsomal nor soluble fraction from brain was capable of restoring the liver sterol biosynthetic activity shown by liver microsomal + soluble preparations.

DISCUSSION

The results herein reported indicate that at present isolated subcellular preparations of brain tissue, from rats 15 days old or adult, do not adequately express the capacity of the tissue to synthesize cholesterol. In all such preparations the synthesis becomes "blocked" at the squalene to cholesterol stage, with the accumulation of large amounts of squalene. The block is not entirely at the squalene cyclization stage, since it has not been possible to detect the conversion of either squalene oxide or lanosterol to cholesterol in vitro. Some blockage may occur at the desmosterol stage. Since some conversion of this sterol to cholesterol was found in vitro, this does not appear to be a major defect. In the intact chick embryo, according to Fish et al. (34) the conversion of desmosterol to cholesterol, involving reduction of the 24,25 double bond, appears to be the rate-determining step in the synthesis of cholesterol. Recent results of Hinse et al. (26) have indicated a maximum of 7.3% conversion of desmosterol to cholesterol in cell-free preparations of 14-day-old rat brain. By 25 days the level of conversion had dropped to 4.8%. Other workers had previously found a lower concentration of desmosterol and a lower level of turnover of desmosterol in adult animals compared to young animals (35,36). Similarly, Holstein et al. (37) have suggested that the conversion of Δ^7- and Δ^8-cholesten-3β-ol compounds to cholesterol by rat brain may be rate limiting. It is not possible at this time to relate these observations to in vitro studies since the major product in the latter case(s) is squalene.

At this stage of our studies it appeared that a review should be made of the methods currently used to study the biosynthesis of cholesterol by the central nervous system. This discussion is presented in the second part of this study (38).

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Studies on the Enzymatic Synthesis of Cholesterol: Use of a Liver Acetone Powder[1]

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ABSTRACT

We have defined special conditions for the preparation of an acetone powder of rat liver microsomes which is capable of converting squalene to cholesterol in high yield. This preparation is also useful for the demonstration of cofactor requirements for certain reactions in sterol biosynthesis. Buffer washed acetone powders are virtually completely dependent upon the 105,000 x *g* supernatant of rat liver (S_{105}) for activity, yet S_{105} by itself is inert in sterol synthesis. The ability of S_{105} to stimulate sterol synthesis is heat liable, nondialyzable, trypsin sensitive, and has been partially purified by ammonium sulfate precipitation and chromatography on Sephadex G-200. These results plus other experiments support the following hypothesis: the 105,000 x *g* supernatant of rat liver (S_{105}) contains a noncatalytic carrier protein (Sterol Carrier Protein or SCP) which originates from the endoplasmic reticulum, binds the substrate, and makes the substrate reactive to the sterol synthesizing enzymes present in the acetone powder of liver microsomes. The participation of SCP may be an important general mechanism in the biological synthesis of cholesterol.

In this report we will review and discuss some of our studies concerning the enzymatic synthesis of cholesterol performed with an acetone powder of rat liver microsomes. We will review briefly (a) the preparation of an acetone powder of rat liver microsomes capable of sterol synthesis, (b) the demonstration of cofactor requirements, and (c) the evidence for a noncatalytic carrier protein in cholesterol biosynthesis (1-3).

Studies by several investigators (4-6) have shown that the enzymes responsible for the conversion of squalene to cholesterol reside in the particulate fraction sedimented at 105,000 x *g* (microsomes). Initial attempts to prepare an acetone powder of rat liver microsomes capable of sterol biosynthesis were unsuccessful (5). However, recently we succeeded in preparing for the first time an acetone powder of rat liver microsomes [supplemented with the 105,000 x *g* supernatant (S_{105}) of rat liver] which was capable of sterol biosynthesis in high yield (7-9). Our success was due to the special conditions used for preparation of the acetone powder. These special conditions are: (a) Spectroquality acetone is redistilled immediately before preparation of the powder. (b) Liver is homogenized using a loose-fitting pestle. (c) The incubations are carried out in phosphate buffer containing a small amount of EDTA. (d) Pyridine nucleotides (NADPH and NAD) are added. (e) S_{105} is added. Using these conditions and squalene-^{3}H as substrate, efficient cholesterol synthesis was observed, and the distribution of products was nearly identical with that observed for a 20,000 x *g* supernatant of rat liver. These results also supported the conclusion that the lipid removed by the acetone and diethyl ether extraction used to prepare the powder is not required for enzymatic activity in these reactions (7-9).

The acetone powder preparation showed an absolute requirement for added NADPH and NAD in the conversion of squalene to sterols (9). In addition, the acetone powder was useful for the demonstration of a pyridine nucleotide requirement (NADP or NAD) in the conversion of Δ^7-cholesten-3β-ol to 7-dehydrocholesterol (10). This was important because in previous studies using microsomal preparations of rat liver, no cofactor requirements (other than oxygen) could be demonstrated for this reaction (11).

In our studies using the acetone powder preparation, we noted that S_{105} was required for maximal activity (9). We found further that the material stimulating sterol synthesis in S_{105} was nondialyzable and destroyed by heat (12). Of crucial significance was the finding that when S_{105} was incubated with squalene-^{3}H in

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the presence of NADPH and NAD, but in the absence of the acetone powder of liver microsomes, no conversion of squalene to sterol was observed (9). Thus, although S_{105} stimulated sterol synthesis, it was inert by itself.

Given these experimental observations and at about the time the acetone powder work was published (9), the following hypothesis occurred to us: S_{105} contains a noncatalytic carrier protein which originates from the endoplasmic reticulum, binds the substrate, and makes the substrate reactive to the sterol synthesizing enzymes present in the acetone powder of liver microsomes (1-3). We have proposed the name Sterol Carrier Protein (SCP) for this substance (3). [Ritter and Dempsey in a recent abstract (Circulation 42: Suppl. 3, III-2, 1970) have independently suggested a slightly different name, i.e., "squalene and sterol carrier protein (SCP)" for a protein found after heating S_{105}. They have partially purified this material (17,18).]

Although the results described above (9) were consistent with a carrier protein hypothesis, the results were for one substrate only, squalene, and the acetone powder by itself was still partially active. We then proceeded to synthesize several additional substrates for examination and performed washing experiments of the acetone powder.

In these experiments acetone powder was washed three times with phosphate buffer by homogenization and centrifugation. The unwashed acetone powder achieved substantial conversion of squalene-^{3}H to sterol. However, the buffer-washed acetone powder was essentially inactive, as was S_{105}; but the combination of the washed acetone powder plus S_{105} converted squalene to sterol in good yield (3). Several other substrates were studied in a similar manner (3). The other reactions studied include: (a) squalene-2,3-oxide→lanosterol (dihydrolanosterol); (b) Δ^7-cholestenol→$\Delta^{5,7}$-cholestadienol; (c) $\Delta^{5,7}$-cholestadienol→cholesterol; and (d) $\Delta^{5,24}$-cholestadienol→cholesterol. The results of these experiments were similar to those obtained with squalene. The buffer washed acetone powder or S_{105} individually have low activity, however, the combination of S_{105} and the washed acetone powder efficiently converts substrate to product.

Additional support for a sterol carrier was obtained by an experiment in which squalene-^{3}H was incubated with an unwashed acetone powder alone (plus cofactors) in the absence of S_{105}. The distribution of products was examined by silicic acid chromatography (9). A similar experiment was done adding S_{105}. Several important differences were noted: (a) More cholesterol was formed in the presence of S_{105} than in the absence of S_{105}. (b) The distribution pattern of intermediate sterols was similar in the two experiments except for greater relative amounts of intermediates in the presence of S_{105} than when S_{105} was absent. (c) Unreacted squalene-^{3}H accounted for a higher percentage of the recovered radioactivity in the absence of S_{105} (3).

These results suggested: (a) Both the acetone powder and S_{105} contain SCP, and SCP is removed from the acetone powder by washing. (b) The SCP present in S_{105} stimulates all of the enzymatic steps involved in the conversion of squalene to cholesterol. Although these results were consistent with the carrier hypothesis, other explanations were possible. It was possible that S_{105} might aid nonspecifically to solubilize the substrates utilized in the study. However, we found that S_{105} could not be replaced by protein materials such as serum albumin or rat serum. In addition, SCP activity in S_{105} is destroyed by trypsin (3). It is significant also that both aerobic reactions (squalene →sterols and Δ^7-cholestenol→$\Delta^{5,7}$-cholestadienol) and anaerobic reactions (squalene-2,3-oxide→lanosterol (dihydrolanosterol), $\Delta^{5,7}$-cholestadienol→cholesterol and $\Delta^{5,24}$-cholestadienol→cholesterol) were stimulated. Thus, since these reactions vary in cofactor requirements (3), it seemed unlikely that S_{105} stimulation could be related to pyridine nucleotide oxidation.

Evidence was obtained (2,3) concerning the ability of S_{105} to bind sterol precursors. Squalene-^{3}H was incubated with S_{105} alone, and then S_{105} was placed on a Sephadex G-25 column. All of the recovered radioactivity was associated with the protein fraction. In a control experiment when squalene-^{3}H was added to buffer, and placed on a Sephadex G-25 column, the radioactivity could not be eluted with buffer and the substrate was recovered from the top of the column by extraction with organic solvents.

S_{105} was placed on a Sephadex G-75 column. An aliquot from the void volume was added to an acetone powder and incubated with Δ^7-cholestenol as substrate. The acetone powder was inactive by itself, however, the acetone powder plus the above aliquot synthesized 7-dehydrocholesterol (3). An additional aliquot was assayed for endogenous cholesterol content by a modification of the Liebermann-Burchard procedure (13). Virtually all of the cholesterol present in S_{105} was recovered in this region (void volume) of the chromatogram. Thus, we detected a protein fraction from Sephadex G-75 that had both SCP activity and

endogenous cholesterol. Since this material traveled at the void volume on Sephadex G-75, we decided to apply a higher resolution technique, i.e., chromatography of S_{105} on Sephadex G-200. Using this technique, SCP activity was present in material retained by the column whereas cholesterol emerged with protein traveling at the void volume. We did not detect the presence of sterol in the protein containing SCP activity. SCP has a mobility on Sephadex G-200 very similar to that of hemoglobin (mol wt 68,000). [Some caution should be used in calculating the molecular weight of SCP from its behavior on Sephadex G-200 since it is possible that the presence of lipids such as phospholipid in SCP might influence its chromatographic mobility on Sephadex.]

SCP was partially purified from S_{105} by precipitation with buffered ammonium sulfate (pH 7.4) between 40-70% saturation and by chromatography on Sephadex G-200. After two passages through Sephadex G-200, only one protein peak was observed. It was then incubated with squalene-^{3}H, and the incubation contents were applied to a Sephadex G-200 column. Two protein peaks were detected, one with the chromatographic mobility of SCP and the other peak with a mobility corresponding to the void volume. The new protein peak traveling at the void volume contained approximately 80% of the added radioactive squalene.

These results are interesting since the protein peak formed after incubation of squalene with SCP has the same chromatographic mobility as the protein peak containing endogenous cholesterol when S_{105} is chromatographed on Sephadex G-200. This suggests that SCP aggregates to a higher molecular weight species in the presence of squalene-^{3}H forming SCP with bound squalene.

Regarding the intracellular localization of this carrier protein we presented evidence (1-3,12) which is consistent with two possibilities. Both the acetone powder and S_{105} contain SCP activity, and the SCP activity can be removed from the acetone powder by washing with phosphate buffer. Thus, the first possibility is that SCP originates from the endoplasmic reticulum and that it is liberated into S_{105} during mechanical rupture of the cell. Consistent with this idea is our observation that the activity of acetone powders alone is somewhat variable (3,12), and that preparations using an intermediate 6,000 x *g* supernatant rather than the 20,000 x *g* supernatant (9) for the preparation of microsomes (for use in making the acetone powder) tend to be more active alone. Alternatively, a second possibility is that SCP may be present in both the endoplasmic reticulum and the cell cytoplasm.

A number of investigators have reported stimulation of sterol synthesis by the 105,000 x *g* supernatant of rat liver (S_{105}), however, the mechanism remained obscure; specifically, Bucher and McGarrahan (4) in the conversion of acetate to cholesterol; Tchen and Bloch in the conversion of squalene to lanosterol (5); Kandutsch in the conversion of 7-dehydrocholesterol to cholesterol (14); Avigan et al. in the conversion of lanosterol to dihydrolanosterol (15); and Dempsey in the conversion of 7-dehydrocholesterol to cholesterol (16). Ritter and Dempsey in recent preliminary communications (17,18) describe a requirement for a heat stable protein derived from the 105,000 x *g* supernatant of rat liver which stimulates the conversion of 7-dehydrocholesterol to cholesterol. In addition this material binds squalene and sterols. In our hands only a small amount of stimulating activity remains after heating S_{105} (2,3,12). The reason for this difference is not apparent. Other investigators have noted that stimulation by S_{105} of various reactions in sterol synthesis is heat liable (15,19).

One might speculate about the chemical makeup of SCP. It is possible that this substance may be a lipoprotein containing phospholipid. The presence of phospholipid in SCP might well facilitate binding of sterols because of the detergent properties of phospholipid. Thus, there may be an important close relationship between lipoprotein synthesis and cholesterol synthesis. Further experiments will be needed to answer these questions.

The experimental results summarized here are consistent with the following hypothesis: S_{105} contains a noncatalytic carrier protein which binds the substrate, and makes the substrate reactive to the sterol synthesizing enzymes present in the acetone powder of liver microsomes (1-3). We have proposed the name Sterol Carrier Protein (SCP) for this substance (3).

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Metabolism of Lathosterol by *Drosophila Pachea*[1,2]

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ABSTRACT

Drosophila pachea is a Sonoran Desert cactiphilic species unable to utilize cholesterol or cholestanol for larval growth and maturation. Lathosterol (7-cholesten-3β-ol) was added to a sterol deficient medium on which an axenic culture of *D. pachea* was maintained. 7-Dehydrocholesterol was identified as the sterol metabolite in adult flies by gas liquid and thin layer chromatography and by its UV spectrum.

INTRODUCTION

The fruit fly, *Drosophila pachea*, was first collected from the Sonoran Desert in northern Mexico by Patterson and Wheeler (1). This species could not reproduce on the standard fruit fly media used and a culture was not established. Later, Heed and Kircher (2) found that *D. pachea* bred only in the rotting stems of a single species of giant cereus cactus, the senita cactus [*Lophocereus schottii* (Englemann) Britton and Rose]. When a cube of fresh, rotted or autoclaved cactus was added to each vial of the common fruit fly medium (bananas, malt, corn syrup, yeast and agar) *D. pachea* could be successfully maintained in continuous culture. Without the added cactus, larvae from oviposited eggs would develop to about the second instar and then die. Addition of other cactus species found in the desert supported the larvae no better than cactus-free control medium (3). This apparent dependence on senita cactus indicated a unique dietary requirement not found in other possible desert breeding sites.

Djerassi et al. (4) reported that senita cactus contained two uncommon sterols, lophenol (4α-methyl-7-cholesten-3β-ol) and schottenol (7-stigmasten-3β-ol). Heed and Kircher (2) isolated these sterols from the cactus and added them to the cactus-free medium. It was found that of the two, schottenol enabled *D. pachea* to develop normally. Eight other sterols were added separately to the medium to ascertain *D. pachea*'s sterol requirements. Lathosterol, 7-dehydrocholesterol and 7-dehydro-β-sitosterol (contaminated with some 7-dehydrocampesterol) fulfilled these requirements. No imagos resulted when cholesterol, β-sitosterol, stigmasterol, 7-ergosten-3β-ol or ergosterol were added to the medium. The females reared from the 7-dehydro-β-sitosterol medium were infertile; they became fertile when placed on senita cactus medium. *D. pachea* was the first insect reported unable to utilize cholesterol for growth and development. This has been recently retested by axenic larval transfers to a sterol deficient medium containing purified cholesterol. Larval death in the second and third instars again occurred (5). These results suggest that *D. pachea* requires Δ^7 or $\Delta^{5,7}$ unsaturated C_{27} or C_{29} sterols, and that it cannot utilize Δ^5 C_{27} or C_{29} sterols, or any of the C_{28} sterols. A second insect with a specialized sterol requirement has been reported. The beetle *Xyloborus ferrugineus* requires ergosterol or 7-dehydrocholesterol for pupation (6).

In this study, lathosterol was fed to axenic *D. pachea* as the major sterol. A single sterol metabolite was isolated from adult files and shown to be 7-dehydrocholesterol.

MATERIALS AND METHODS

Axenic Culture

An axenic (microbiologically sterile) culture of *D. pachea* was obtained by a modification of the peracetic acid egg sterilization method of Doll et al. (5,7). This culture was maintained axenically for many generations. The sterility was checked periodically by transferring a portion of used media into fluid thioglycollate and trypticase soya broths. Only flies from microorganism-free populations were used for sterol analysis.

Diet

The medium used throughout the experiment is shown in Table I.

To determine residual sterol, the ingredients for a liter of medium were hydrolyzed with alcoholic KOH and the nonsaponifiables were

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the medium was not responsible for the $\Delta^{5,7}$ dienol UV absorption bands, the benzene fraction from the column was reduced with hydrogen in ethyl acetate over 5% Pd on carbon for 3 1/2 hr. Authentic 7-dehydrocholesterol and ergosterol were reduced in a similar manner.

RESULTS

The 1:1 benzene-hexane and ether fractions from the column showed no UV absorption characteristic of a conjugated system. The benzene fraction showed end absorption at 207 nm with an absorption pattern suggestive of a $\Delta^{5,7}$ diene, but at wavelengths lower than authentic 7-dehydrocholesterol, 258, 280 and 294 nm. The spectrum of the digitonide of this fraction was in exact agreement with the digitonide of 7-dehydrocholesterol, with absorption at 261, 272, 282 and 294 nm.

TLC also suggested the presence of 7-dehydrocholesterol. The 1:1 benzene-hexane fraction showed only a faint spot for this compound whereas the benzene fraction gave a dark spot identical in R_f to that of 7-dehydrocholesterol. Both fractions also contained lathosterol, which had double the R_f of 7-dehydrocholesterol on argentation TLC.

GLC of the 3 sterol containing fractions from the column on three different liquid phases gave a single peak in each case corresponding in relative retention time (RRT) to lathosterol and 7-dehydrocholesterol (Table II). The two sterols could not be separated by any phase. No other sterols were detected. Cholestanol, cholesterol, ergosterol and 7-ergosten-3β-ol were all well resolved from the lathosterol-7-dehydrocholesterol peak.

The reduction products of the benzene fraction were further evidence for the absence of ergosterol in the fly sterols. Only two peaks were obtained from the reduced mixture. These corresponded to cholestanol and lathosterol (Table II). No peaks corresponding to reduced ergosterol compounds were observed.

All of the evidence is in support of the thesis that *D. pachea* can convert dietary lathosterol to 7-dehydrocholesterol by introducing unsaturation at Δ^5 in the sterol nucleus.

DISCUSSION

The sequence of double bond changes in ring B during the biosynthesis of cholesterol in animals is in the order $\Delta^8 \rightarrow \Delta^7 \rightarrow \Delta^{5,7} \rightarrow \Delta^5$ (13). The last two steps in this process have been shown to be irreversible in mammalian systems (14). In insects, however, an overall $\Delta^5 \rightarrow \Delta^{5,7}$ transformation has not only been demonstrated (15,16), but may be obligatory in all species ingesting Δ^5 sterols because the moulting hormones are 6-keto-Δ^7 derivatives (17).

A study of sterols in crickets led Martin and Carls (18) to postulate a sequence of double bond changes in insects ($\Delta^5 \rightarrow \Delta^0 \rightarrow \Delta^7 \rightarrow \Delta^{5,7}$) based on their observations that Δ^7 appears to be metabolically closer to $\Delta^{5,7}$ than Δ^5 is. The intermediary role of cholestanol (Δ^0) between Δ^5 and Δ^7 was also suggested by the $\Delta^0 \rightarrow \Delta^7$ transformation found in two roaches (19). The desaturation of cholestanol to cholesterol has never been reported in insects and it has not been established that insects can introduce Δ^5 unsaturation in the sterol molecule.

To our knowledge there has been no prior study of the metabolism of lathosterol in insects. Our findings that this sterol is metabolized to 7-dehydrocholesterol by *D. pachea* lends credence to the last step of the pathway suggested by Martin and Carls. If the general metabolic pathway of sterol conversions in insects is $\Delta^5 \rightarrow \Delta^0 \rightarrow \Delta^7 \rightarrow \Delta^{5,7}$, it appears that since *D. pachea* cannot complete its life cycle on cholesterol, it lacks the biochemical ability to convert cholesterol to lathosterol. This may be either at the $\Delta^5 \rightarrow \Delta^0$ step or the $\Delta^0 \rightarrow \Delta^7$ step. Our recent work (H.W. Kircher and M.A. Gray, unpublished work) has shown that *D. pachea* cannot utilize cholestanol either, thereby ruling out the former possibility and showing that the $\Delta^0 \rightarrow \Delta^7$ pathway is blocked. This latter case is not uncommon in insects; neither hide beetles, houseflies nor silk worms are able to utilize cholestanol as the sole dietary sterol (19-21). *D. pachea*, unable to utilize cholesterol or cholestanol, converts dietary lathosterol to 7-dehydrocholesterol. The latter sterol is also found as a metabolic product when cholesterol is administered to roaches and houseflies (15,16).

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TABLE II

Percentage Composition of the Glyceryl Alk-1'-enyl Ether Aldehydes in the Phospholipid Fractions of Whole Brains From Germ-free and Conventional Rats[a]

Lipids isolated from whole brain tissues	Hydrocarbon chain, wt %							
	14:0	15:0	16:0	16:1	17:0	18:0	18:1	18:2 or 19:0
Germ-free rat	Trace	Trace	21.4	Trace	1.2	39.1	38.6	Trace
Conventional rat	Trace	Trace	19.3	Trace	1.4	41.3	38.2	Trace

[a]Values given are mean of triplicate determinations of duplicate biological samples.

ethers were determined quantitatively by the densitometric method of Privett et al. (13). Larger quantities of alk-1'-enyl glyceryl ethers were obtained by preparative TLC. The plates were developed in the previously mentioned solvent system and the alk-1'-enyl glyceryl ethers were visualized by spraying with a solution of 0.2% 2',7'-dichlorofluorescein in ethanol; the lipid areas were outlined under UV light. Cleavage of the zones containing the alk-1'-enyl glyceryl ethers were achieved by the method of Anderson et al. (14). The aldehyde composition of the alk-1'-enyl glyceryl ethers from conventional and germ-free rat brain was then determined by gas liquid chromatography at 170 C on an EGSS-X 6 ft column.

The percentage of alkyl and alk-1'-enyl glyceryl ethers in total brain tissue of conventional rats has been determined by several groups of investigators. The values obtained using different procedures for these determinations vary from 5% alk-1'-enyl glyceryl ether of the isolated phosphatides (12) to 25% (15). This variation has been attributed to the age, diet, etc. of the animals (12).

We have found that determination of the alk-1'-enyl glyceryl ether content of several bacteria and tissues (unpublished observation) by reduction of phosphatides with $LiAlH_4$, followed by the extraction of the reaction products with ether, gives slightly lower values for this lipid content than other methods (16). The values for alk-1'-enyl glyceryl ether content of conventional rat brain tissue reported here are lower than those reported by Erickson and Lands (12% and 25% respectively) (15), but considerably higher than the 5% reported by Wood and Snyder (12).

The results (Table I) show, however, that when the brain lipids of conventional and germ-free rats are treated identically and the amount of ether-containing lipids is determined by the same method, no difference can be detected in the amount of ether-containing lipids from these tissues. It is further shown (Table II) that there is no detectable difference in the composition of the fatty aldehydes isolated from the plasmologens of these two tissues.

From these results, it is apparent that the microbial flora of the rat do not influence the quantity of the ether-containing lipids or the composition of the aldehydogenic chain of the alk-1'-enyl glyceryl ethers in the rat brain.

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Glyceryl Ether Containing Lipids of Whole Brains From Germ-Free and Conventional Rats

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ABSTRACT

The percentage alkyl and alk-1'-enyl glyceryl ethers of the polar lipids isolated from germ-free and conventional rat brain tissue has been determined. No difference could be detected in the quantity of the ether-containing lipids in these animals. Similarly, the composition of the aldehydic chain of the alk-1'-enyl glyceryl ethers from germ-free and conventional rat brain tissue was shown to be the same. The microbial flora, therefore, do not influence either the quantity of the ether-containing lipids or the composition of the aldehyde chain of the alk-1'-enyl glyceryl ethers in the rat brain.

The presence of alkyl and alk-1'-enyl glyceryl ether in mammalian tissues has been recognized for several years (1). The biosynthesis of these lipids, however, is still not fully understood although recent reports indicate that alkyl ethers can be synthesized by both normal and malignant cells from dihydroxyacetone phosphate and long chain fatty alcohols (2,3), and that alkyl ethers may be the precursor of alk-1'-enyl glyceryl ethers (4). Most anaerobic bacteria (5) appear also to contain substantial quantities of alk-1'-enyl glyceryl ether. Alkyl glyceryl ethers have recently been identified in *Clostridium butyricum* (6).

Both in mammalian cells and in bacteria, the aldehydogenic chain of the alk-1'-enyl glyceryl ethers is composed mainly of 16:0, 18:0 and 18:1 hydrocarbon moieties (1) which, in the case of mammalian cells, is somewhat different from the composition of the ester-linked chains in the 1 position of the phosphatides (1). The major aldehydogenic chain of ox-spleen and ox-liver alk-1'-enyl glyceryl ethers has been identified as branched-chain 15-carbon aldehyde (7) while rumen bacteria has been shown to synthesize appreciable quantities of higher branched-chain aldehydes including a branched-chain 15-carbon aldehyde (8). This observation led us to investigate the possible involvement of bacterial systems in the synthesis of mammalian ether-containing lipids. The result presented here rules out such involvement.

Lipids were extracted as previously described (9) from whole brains of adult female rats (local strain, 170 g) and female germ-free adult rats (Charles River CDRF strain, 158 g), which had been maintained on the same diet. The germ-free rats were reared according to the technique described by Midtvedt and Trippestad (10). Nonlipid contaminants of the lipid extract were removed by washing with 0.05 N sodium chloride as described by Folch et al. (11). Neutral lipids and phospholipids were separated on a silicic acid column (9); the neutral lipids were eluted with chloroform and the phospholipids with methanol. The phospholipids were reduced by lithium aluminum hydride as described by Wood and Snyder (12), and the alkyl and alk-1'-enyl glyceryl ethers were separated by thin layer chromatography (TLC) on Silica Gel G using diethyl ether-water (100:0.5 v/v). Alkyl and alk-1'-enyl glyceryl

TABLE I

Alkyl- and Alk-1'-enyl-Glyceryl Ether Concentrations in the Phospholipid Fractions of Whole Brains From Germ-Free and Conventional Rats[a]

Lipid isolated from whole brain tissue	Percentage of total phospholipid[b]		
	Alk-1'-enyl-glyceryl ether	Alkyl-glyceryl ether	Ratio[c]
Germ-free rat	4.25 ± 0.21	0.75 ± 0.04	5.66
Conventional rat	4.63 ± 0.28	0.82 ± 0.06	5.63

[a]Values given are the mean of triplicate determination of duplicate biological samples.
[b]Percentages given are expressed as free O-alkylglycerols.
[c]Ratio of alk-1'-enyl- to alkyl-glyceryl ether.

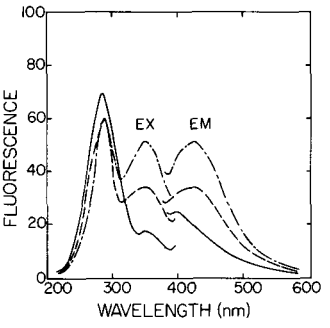

FIG. 3. Fluorescence of peroxidized HSA. Freshly prepared HSA was allowed to peroxidize for 0 hr EX set at 350 nm, EM set at 425 nm (—); 168 hr EX 345 nm, EM 425 nm (– –); and 240 hr EX 350 nm, EM 425 (– • –).

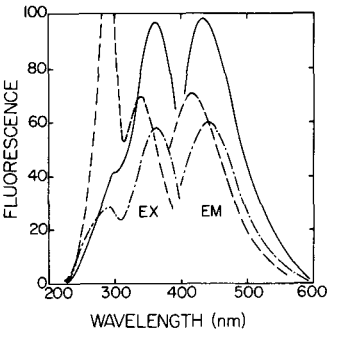

FIG. 4. Fluorescence of reaction products of BSA and carbonyls. BSA was reacted with 2,4-hexadienal EX 360 nm, 435 nm (—); crotonaldehyde EX 340 nm, EM 420 nm (– –); and 2,3 butanedione EX 360 nm, EM 440 nm (– • –).

Reaction of Peroxidizing Methyl Linoleate With BSA

Methyl linoleate, 22 mg, and BSA, 100 mg, were mixed together in 100 ml of distilled water. The mixture was shaken to disperse the oil and then was allowed to peroxidize at 25 C. A few drops of toluene were added as a bacteriostatic agent. Aliquots were withdrawn at various time intervals and diluted with an equal volume of distilled water before recording the fluorescence spectra.

Reaction of Carbonyls With BSA

BSA, 25 mg (9.2×10^{-7} mole) in 25 ml of 0.05 M sodium phosphate buffer, pH 7.0, was reacted with 1.2×10^{-4} mole of 2,4-hexadienal, 2,3-butanedione or crotonaldehyde. The mixtures were stirred at 25 C for 25 hr and were then dialyzed for 24 hr against distilled water to remove the carbonyl compounds. After adjustment of the protein concentration to 1 mg/ml, the fluorescence spectra were recorded. Protein determinations were carried out by the method described by Miller (3).

Preparation and Peroxidation of HSA

The albumin fraction of serum freshly separated from whole human blood was obtained by the cold methanol preparation described by Straumfjord and Spraberry (4). The albumin fraction was dialyzed against distilled water. Fluorescence measurements were made before and after dialysis. This freshly prepared HSA (1.35 mg/ml) was allowed to peroxidize at 25 C; toluene was added as a bacteriostatic agent. At various time intervals, aliquots were withdrawn and fluorescence measurements were made.

TABLE I

Fluorescence of Commercial Human Serum Albumin[a]

Type preparation and/or grade	Source[b]	Fluorescence: Excitation, nm	Fluorescence: Emission, nm	Relative intensity[c]
Fraction V	NBC	348	425	31
Crystallized 4X	NBC	350	426	62
Grade III	Sigma	348	420	26
Crystalline, 100% by electrophoresis	Mann	348	430	78
Fraction V	Mann	348	430	19
Fraction V	Calbiochem	348	430	24
Crystalline, B grade	Calbiochem	344	428	76

[a]Protein concentration was 1 mg/ml in 0.05 M sodium phosphate buffer, pH 7.0.

[b]NBC, Nutritional Biochemicals Corp.; Sigma, Sigma Chemical Co.; Mann, Mann Research Laboratories; Calbiochem, California Biochemical Corp.

[c]Fluorescence intensity of 0.1 μg quinine sulfate per milliliter of 0.1 N H_2SO_4 set at 100.

Fluorescent Modification of Serum Albumin by Lipid Peroxidation

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ABSTRACT

Peroxidation of fatty acids bound to human serum albumin results in the production of fluorescent chromophores in the protein when it is stored in the liquid, powdered or crystalline state. Peroxidizing polyunsaturated fatty acid esters, and carbonyls derived from peroxidizing lipids react with amino groups of protein to give products that have fluorescence spectra very similar to those observed for stored commercial preparations of serum albumin.

INTRODUCTION

One of the main functions of serum albumin is to bind and transport anionic substances, such as fatty acid anions, needed for metabolic processes throughout the body. The lipids associated with human serum albumin are so tightly bound that fractionated as well as crystallized albumin preparations contain variable amounts of fatty acids (1).

Previous work in this laboratory has shown that carbonyls derived from peroxidizing lipids react with protein to produce fluorescent chromophores (2). Human serum albumin (HSA) is the plasma protein most likely to be susceptible to development of fluorescent modification by lipid peroxidation and this protein was found to be the most fluorescent of the plasma proteins. In this work the observed fluorescent modification in stored HSA was correlated with that found in model peroxidizing lipid-protein systems.

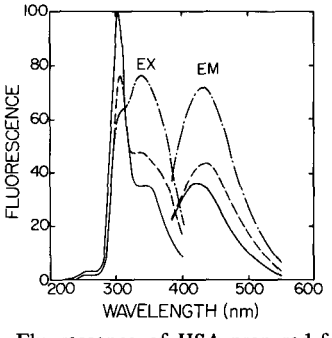

FIG. 1. Fluorescence of HSA prepared for clinical use. Protein concentration was adjusted to 10 mg/ml after dialysis against distilled water for 48 hr. Fluorescence spectra represent HSA prepared by the following laboratories: Pitman-Moore EX 340 nm, EM 435 nm (– • –); Hyland EX 345 nm, EM 435 nm (– –); and Cutter EX 350 nm, EM 425 nm (——).

MATERIALS AND METHODS

Solutions of 5% HSA in buffered saline suitable for therapeutic injection were obtained from Pitman-Moore and from Hyland Laboratories, and a 25% solution of salt-poor HSA from Cutter Laboratories. Bovine serum albumin (BSA), crystallized and 100% pure as determined by electrophoresis, was obtained from Mann Research Laboratories. Whole human blood units were obtained from the Sacramento Blood Bank, California. Crotonaldehyde and 2,3-butanedione were from Eastman Organic Chemicals; 2,4-hexadienal from K & K Laboratories; and methyl linoleate from Analabs, Inc. All other chemicals used were of reagent grade.

Fluorescence Measurements

An Aminco-Bowman spectrophotofluorometer (American Instrument Co.) combined with a Houston Instrument X-Y recorder was used to make all fluorescence measurements. Quinine sulfate (0.1 and 1.0 μg/ml in 0.1 N H_2SO_4) was used for intensity calibration and as a check on wavelength calibration of the spectrophotofluorometer. Fluorescence excitation (EX) and emission (EM) spectra are reported without correction.

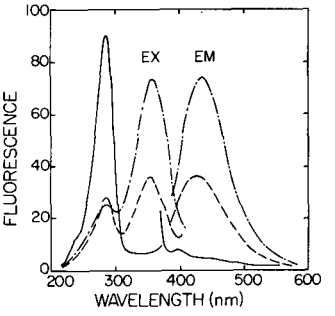

FIG. 2. Fluorescence of BSA reacted with peroxidizing methyl linoleate. Fluorescence spectra were taken at 0 hr EX set at 350 nm, EM set at 435 nm (——); 170 hr EX 355 nm, EM 430 nm (– –); and 264 hr EX 357 nm, EM 435 nm (– • –).

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RESULTS AND DISCUSSION

The excitation and emission maxima and the relative fluorescence intensitites of commercially prepared HSA preparations are shown in Table I. The intensity of fluorescence varied markedly from preparation to preparation but the fluorescence excitation and emission spectra were very similar and wavelength maxima were consistently in the 350 nm and 425 nm regions, respectively. Characteristic fluorescence spectra of HSA commercially prepared for therapeutic use are presented in Figure 1. Again, the fluorescence intensity of each commercial albumin preparation varied from the others, but the excitation and emission maxima were similar.

Figure 2 shows the fluorescence produced when peroxidizing methyl linoleate was allowed to react with a commercial preparation of BSA. Methyl linoleate or BSA alone, when maintained under the same conditions, did not develop similar fluorescence.

When freshly isolated HSA alone was allowed to peroxidize it gave fluorescence spectra, as shown in Figure 3, which were qualitatively very similar to that reported in Table I and shown in Figures 1 and 2 for commercial serum preparations. As can be seen in Figure 3 the freshly isolated or Ohr HSA has little if any fluorescence with excitation at 350 nm and emission at 425 nm. Freshly prepared albumin from bovine and rat sera also show very little fluorescence in this region.

When α, β-unsaturated and α-dicarbonyls were allowed to react with BSA, fluorescent products were produced rapidly. The fluorescence spectra of these products, as can be seen in Figure 4, were very similar to those observed in the commercial preparations and in the model peroxidation systems.

The presence of free fatty acids in Cohn fraction V HSA has been shown by electrophoretic and low temperature solvent extraction studies (5). Of the total fatty acids bound to HSA, 66% was found to be unsaturated, with the major components distributed as follows: oleic acid, 33%; linoleic acid, 20%; arachidonic acid, 5%; and linolenic acid, 1.4%. The association of unsaturated fatty acids with albumin renders both clinical and commercial preparations of this protein quite labile to peroxidation by air, as reported here and in previous studies (2,6).

Secondary degradation products of peroxides produced by lipid peroxidation include a wide range of mono- and dicarbonyls, both saturated and unsaturated (7,8). Carbonyls condense with primary amines to form a Schiff-base imine system. Previous studies (2) have shown that peroxidizing lipids and the di-carbonyl malonaldehyde react to produce inter- and intramolecular cross-linking or polymerization of ribonuclease A with concurrent inactivation of the enzyme and the production of fluorescence similar to that found here. Three of the major classes of carbonyls produced in peroxidizing unsaturated lipid systems react with primary amines to produce fluorescent products. These carbonyls are the conjugated α, β mono- and diene-aldehydes, and the α-dicarbonyls. It is assumed that the reaction is carbonyl-amine condensation, with the production of a conjugated chromophoric Schiff-base system (2).

Freshly isolated serum albumin is colorless in both the dry state and in solution. Upon storage, however, both forms of HSA develop a yellow to brown color which is characteristic of peroxidizing lipid-amine reaction systems. Deterioration of both HSA and BSA during storage has been reported by Pederson (9). By use of exclusion chromatography, it was found that dimers, trimers and tetramers of albumin were formed upon storage of the protein in solution. Further research is necessary to determine the quantity of the cross-linking of albumins that results from the reaction of dicarbonyls with protein and that which occurs by various other mechanisms, such as sulfhydryl cross-linking.

The physiological effects of administration of modified HSA to patients are unknown. Anionic binding capacity may be altered in the modified protein. The modified portions of albumins may be resistant to proteolytic hydrolysis by systems such as the liver lysosomes. These modified proteins could further polymerize and remain within the lysosomes to hamper the efficiency of the organelle. Similarly, intravenous injections of exogenous lipid give rise to a fat pigment which is associated with the lysosomes of Kupffer cells and hepatocytes. The pigment is thought to consist of polymerized lipid-containing material; it is unhydrolyzable by the organelle and remains fixed for the duration of the animal's life (10).

ACKNOWLEDGMENT

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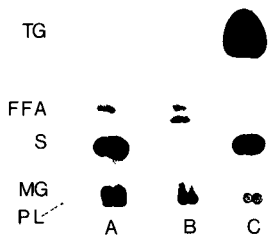

FIG. 1. A, sporophore total lipids; B, mycelial total lipids; C, triolein and cholesterol. PL, polar lipid; MG, monoglyceride; S, free sterol; FFA, free fatty acid; TG, triglyceride (The TG spot in fractions A and B formed faint spots not visible in this photograph).

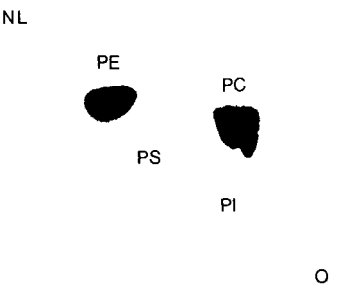

FIG. 2. Two-dimensional separation of sporophore polar lipids. O, origin; PI, phosphatidyl inositol; PS, phosphatidyl serine; PC, phosphatidyl choline; PE, phosphatidyl ethanolamine; NL, neutral lipid (PI, PS and NL formed very faint spots and were not visible on this photograph).

Thin Layer Chromatography

The neutral lipid fraction was chromatographed on glass plates coated with Silica Gel G. Acc. to Stahl (Merck) in a solvent system of low-boiling petroleum (bp 60-70 C)-diethyl ether-acetic acid (90:10:1 v/v). The polar lipids were chromatographed in two dimensions on Silica Gel H (Merck) as described by Parsons and Patton (7). A solvent system of chloroform-methanol-water-28% aqueous ammonia (130:70:8:0.5 v/v) was used in the horizontal development and chloroform-acetone-methanol-acetic acid-water (100:40:20:20:10) was used in the vertical development. The spots were made visible by exposure to iodine vapor or by spraying with one of the following reagents: sulfuric acid-potassium dichromate (8), the specific phospholipid spray of Dittmer and Lester (9), and ninhydrin reagent (0.2% in ethanol) for compounds containing amino groups. Individual polar lipids were identified by their reaction with specific spray reagents and by comparing their Rf values with those of reference standards and polar lipid extracts from bovine milk.

Gas Liquid Chromatography

Fatty acid methyl esters were made by saponification of the neutral and polar fractions in 0.5% KOH in methanol and refluxing in BF_3-methanol (Applied Science Laboratories). Methyl esters were extracted and chromatographed on a Hewlett-Packard 5750 Gas Chromatograph equipped with a hydrogen flame ionization detector. Separations were effected with a column packed with 10% diethylene glycol adipate containing 2% H_3PO_4 on Gas Chrom A (Applied Science). Retention times of unknown methyl esters were compared with those of authentic methyl esters to make a tentative identification. Quantitative standards K-106 and K-108 (Applied Science) were used to determine the linearity of peak area with concentration. The standard deviations of the concentrations as determined by measuring peak area (height x width at 1/2 height) were not greater than ± 1.6%.

Gas Liquid Chromatography-Mass Spectrometry

Identification of fatty acid methyl esters was accomplished on an LKB Model 9000 Gas Liquid Chromatograph-Mass Spectrometer equipped with a chromatographic column of the packing material previously described. The peaks from sporophore mycelial extracts with retention times equal to palmitic, stearic, oleic and linoleic acid methyl esters were superimposable upon spectra from authentic fatty acid methyl esters.

Infrared Spectrophotometry

IR spectra were recorded on a Perkin-Elmer Model 137. Infracord Spectrophotometer. The samples were analyzed as thin films on KBr discs.

RESULTS

The percentage contributions to the total lipid by the neutral and polar fractions for sporophore and mycelium is shown in Table I.

TABLE I

The Per Cent of the Neutral and Polar Lipids in *A. Bisporus*[a]

Strain	Sporophore extract	Mycelial extract
310	32.6 Neutral 67.3 Polar	66.1 Neutral 33.8 Polar
314	40.3 Neutral 59.6 Polar	54.3 Neutral 45.6 Polar
320	51.1 Neutral 48.7 Polar	79.2 Neutral 20.8 Polar
322	49.4 Neutral 50.3 Polar	76.3 Neutral 23.6 Polar

[a]Values are expressed as per cent of total.

Lipid Metabolism of *Agaricus bisporus* (Lange) Sing.: I. Analysis of Sporophore and Mycelial Lipids[1]

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ABSTRACT

The lipid components of four strains of *Agaricus bisporus* (Lange) Sing., the cultivated mushroom, were analyzed. Both sporophore and mycelial samples were obtained from beds in normal production. A method for obtaining mycelium free of compost was developed. Neutral lipids were separated from polar lipids by silicic acid column chromatography. Each fraction was separated by thin layer chromatography. Fatty acid methyl esters were analyzed by gas liquid chromatography and mass spectrometry. Sporophore extracts contained free sterol, free fatty acid, triglycerides, phosphatidyl choline and phosphatidyl ethanolamine. High amounts of linoleic acid were found in both neutral and polar lipid fractions. Mycelial extracts contained free fatty acids, triglycerides, phosphatidylcholine and phosphatidyl ethanolamine. No free sterol could be detected. Linoleic acid was also present in large amounts.

INTRODUCTION

A preliminary analysis of the lipid components of the sporophore of *Agaricus bisporus* (Lange) Sing., the cultivated mushroom, was conducted by Hughes (1). He reported a large amount of free sterol, smaller quantities of sterol ester, triglyceride, free fatty acids and phospholipids. Ten fatty acids were identified and linoleic acid was found to account for 63% to 74% of the amount of fatty acids present, depending on the mushroom variety. Maggioni et al. (2) reported that the total lipid fraction of sporophores growing on nitrogen supplemented compost contained 71% linoleic acid and 15% palmitic acid.

It has been shown that mushroom production increased when lipid material was added to compost (3), and that mushroom mycelial growth was increased when lipids were added to basal nutrient media (4). To better understand lipid metabolism in *A. bisporus*, a more detailed analysis of the lipid components was felt desirable and the present study was initiated. Lipid components of both sporophore and mycelium were analyzed. Neutral and polar lipids were separated. Strains 310 (white), 314 (off white), 320 (brown) and 322 (off white) were compared. Strains 310, 320 and 322 are used in commercial spawn making and account for at least 70% of the mushrooms produced in the United States and Canada. Strain 314 was included as it was the strain which failed to respond with increased mycelial growth to lipid addition.

MATERIALS AND METHODS

Isolation of Sporophore and Mycelial Lipids

Strains 310, 314, 320 and 322 were obtained from the culture collection of The Pennsylvania State University. The compost was prepared as described by Schisler (3) without the addition of any supplementary nutrients at casing. Mycelial samples were harvested by a special method designed to get mycelium free from compost material. Compost was filled into wooden trays (61 x 61 x 14 cm). When filled the trays contained 25-28 kg of compost. Trays were filled with compost and spawned (seeded with mushroom mycelium) to one half their depth. A layer of fiberglass fly screen was placed on top of the compost. A 1 cm layer of water saturated Superlite (Perlite Products, Primos, Pa.) was spread on top of the screening and covered by a second piece of screening. The tray was then filled and spawned to its capacity. The screens were removed from the compost after three weeks. The Superlite clinging to the screen due to the mycelial growth was scraped off into flasks and extracted. A portion of the same compost was spawned for sporophore harvest. After three weeks spawn growth the trays were cased (covered with a layer of topsoil to induce fruiting). Mushrooms were harvested from the first fruiting. Lipids in both sporophore and mycelial samples were extracted according to the method of Folch et al. (5). The neutral lipid fraction was separated from the polar lipid fraction using a silicic acid column as described by Hirsch and Ahrens (6).

[1]Paper 3798 in the Journal Series of The Pennsylvania Agricultural Experiment Station.

TABLE III

Fatty Acid Composition of the Neutral and Polar Lipids of Mycelium[a]

Acid	Strain 310	Strain 314	Strain 320	Strain 322
Neutral lipids				
C10:0	4.1	t	12.1	t
C12:0	5.2	14.6	6.2	7.8
C14:0	8.3	2.9	t	5.2
C16:0	22.1	25.0	19.6	27.6
C16:1	6.6	4.3	9.8	6.1
C17:0	t	10.4	9.0	9.0
C18:0	10.0	7.5	6.7	8.4
C18:1	16.9	18.7	18.6	16.2
C18:2	26.3	16.2	17.6	19.4
Polar lipids				
C10:0	t	t	t	t
C12:0	t	2.0	2.4	1.8
C14:0	t	6.8	5.1	2.4
C16:0	18.8	16.7	18.0	15.2
C16:1	8.5	8.8	6.7	3.7
C17:0	5.1	4.7	3.8	4.5
C18:0	3.7	4.5	2.8	5.5
C18:1	14.1	13.7	12.5	11.1
C18:2	49.8	42.4	48.4	55.3

[a]Values are expressed as per cent of total. Those values marked (t) are less than 1% of the total.

high proportion of linoleic acid. Shaw found that the polar lipid contained most of the linoleic acid.

The results of this study confirmed those of Shaw; however, the advantage of the study reported here was that a valid comparison of mycelial and sporophore lipids of a single species was possible. Sporophores and mycelia were harvested in normal production under a controlled environment and not in vitro on artificial media. It was not necessary to correlate data from diverse species to compare mycelial to sporophore lipids.

Bentley et al. (11) reported that the ratio of neutral lipid to polar lipid in the fruiting body of *Clitocybe illudens* was 1:2 and that the neutral fraction was primarily free sterol. It can be seen in Table I that of the four strains of *A. bisporus* used in this study only strain 310 had a similar ratio of neutral to polar lipids in the fruiting bodies. Free sterol was, however, the predominant lipid in the sporophore neutral fraction.

Utilization of lipid by *A. bisporus* has been shown to be a key factor in the nutrition of the commercial mushroom. Schisler (3) showed that lipid supplementation to compost resulted in increased sporophore yield. Wardle and Schisler (4) showed that lipid additions to basal media gave increased mycelial growth. Ethyl esters of oleic and linoleic acids were shown to be primary stimulatory components.

It is interesting to speculate on the implications of some of the differences in lipid composition of the mycelium and sporophore as found in this study on the lipids of *A. bisporus*. Distinct differences in the weight per cent of the neutral and polar lipids between the sporophore and the mycelium were shown (Table I). The absence of sterol in the mycelium and its appearance in quantity in the sporophore suggests that free sterol is the end product of synthetic events during the fruiting process.

The fatty acids of the sporophore extracts from the various strains were consistent in profile, with the exception of 314 which was significantly lower in linoleic acid than the other three strains. This low level of linoleic acid was concomitant with the fact that strain 314 was giving a low crop yield at the time of sampling. Wardle and Schisler (4) also reported that the growth of mycelium of strain 314 was not increased by ethyl-linoleate supplement. The inability of this strain to utilize linoleic acid may be a cause for low yield. Minor differences in fatty acid composition between strains found in this study are probably attributable to the differences in the metabolism of each strain.

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TABLE II

Fatty Acid Composition of the Neutral and Polar Lipids of Sporophores[a]

Acid	Strain 310	Strain 314	Strain 320	Strain 322
Neutral lipids				
C10:0	t	---	---	---
C12:0	t	t	t	t
C14:0	t	4.9	t	t
C16:0	13.2	26.5	16.3	15.4
C16:1	t	t	t	t
C18:0	4.1	7.4	6.7	2.5
C18:1	5.4	12.2	8.9	3.3
C18:2	77.1	49.0	68.0	78.6
Polar Lipids				
C10:0	t	---	---	---
C12:0	t	t	t	t
C14:0	t	t	t	t
C16:0	6.1	9.2	6.5	5.7
C16:1	t	t	---	---
C18:0	2.0	2.3	1.4	1.8
C18:1	t	t	---	t
C18:2	91.3	88.5	92.0	92.4

[a]Values are expressed as per cent of total. Those values marked (t) are less than 1% of the total.

The ratio of neutral to polar lipids was higher in the mycelium than the sporophore.

A thin layer separation of sporophore neutral lipids showed that free sterol and monoglycerides were the major lipid classes. Smaller amounts of free fatty acids and triglycerides were observed (Fig. 1-A). The areas with Rf values equal to triglycerides, free fatty acids and monoglycerides were scraped from a thin layer separation of neutral lipids. IR spectra of the eluates were similar to reference standard compounds. Upon saponification and esterification of the triglyceride and monoglyceride fractions, fatty acid methyl ester patterns similar to those of the neutral lipids were established by gas chromatography.

Mycelial neutral lipids contained free fatty acids, triglycerides and monoglycerides. No free sterol was detected (Fig. 1-B).

Phosphatidyl choline and phosphatidyl ethanolamine were the predominant polar lipid classes (Fig. 2). The compounds had similar Rf values on two-dimensional thin layer chromatography (TLC) as phosphatidyl choline and phosphatidyl ethanolamine from bovine milk polar lipids and authentic reference compounds. Both compounds reacted with the Dittmer-Lester spray. The compound with an Rf value equal to phosphatidyl ethanolamine reacted with ninhydrin. The IR spectra of the two compounds were superimposable on those of authentic phosphatidyl choline and phosphatidyl ethanolamine. Small amounts of phosphatidyl serine and phosphatidyl inositol were tentatively identified in sporophore polar lipids having similar Rf values as authentic reference compounds and of those components in bovine milk polar lipids. Both reacted with the Dittmer-Lester spray. Neither of these compounds was detectable in mycelial polar lipids. However, the mycelial polar lipids had distribution of phosphatidyl choline and phosphatidyl ethanolamine identical to the sporophore polar lipids.

A typical percentage composition of fatty acids in sporophore neutral and polar lipids was tabulated (Table II). Palmitic, stearic, oleic and linoleic acids accounted for over 90% of the fatty acids present. In the neutral lipids of sporophores, linoleic acid accounted for approximately 70% of the fatty acids. Strain 314, however, had only 49% linoleic acid. Mycelial neutral lipids showed more varied fatty acid compositions than did sporophore neutral lipids (Table III).

The polar lipid extracts of both sporophore and mycelium were predominantly palmitic and linoleic acid (Tables II and III). The sporophore polar fraction was high in linoleic acid, averaging about 90%, whereas the mycelial polar lipids average about 50% of this acid.

DISCUSSION

The lipid components of several basidiomycetes were investigated by Shaw (10). He collected sporophores growing in the wild and reported that fruiting bodies and mycelia had a

TABLE III

Fatty Acid Composition of the Neutral and Polar Lipids of Mycelium[a]

Acid	Strain 310	Strain 314	Strain 320	Strain 322
Neutral lipids				
C10:0	4.1	t	12.1	t
C12:0	5.2	14.6	6.2	7.8
C14:0	8.3	2.9	t	5.2
C16:0	22.1	25.0	19.6	27.6
C16:1	6.6	4.3	9.8	6.1
C17:0	t	10.4	9.0	9.0
C18:0	10.0	7.5	6.7	8.4
C18:1	16.9	18.7	18.6	16.2
C18:2	26.3	16.2	17.6	19.4
Polar lipids				
C10:0	t	t	t	t
C12:0	t	2.0	2.4	1.8
C14:0	t	6.8	5.1	2.4
C16:0	18.8	16.7	18.0	15.2
C16:1	8.5	8.8	6.7	3.7
C17:0	5.1	4.7	3.8	4.5
C18:0	3.7	4.5	2.8	5.5
C18:1	14.1	13.7	12.5	11.1
C18:2	49.8	42.4	48.4	55.3

[a]Values are expressed as per cent of total. Those values marked (t) are less than 1% of the total.

high proportion of linoleic acid. Shaw found that the polar lipid contained most of the linoleic acid.

The results of this study confirmed those of Shaw; however, the advantage of the study reported here was that a valid comparison of mycelial and sporophore lipids of a single species was possible. Sporophores and mycelia were harvested in normal production under a controlled environment and not in vitro on artificial media. It was not necessary to correlate data from diverse species to compare mycelial to sporophore lipids.

Bentley et al. (11) reported that the ratio of neutral lipid to polar lipid in the fruiting body of *Clitocybe illudens* was 1:2 and that the neutral fraction was primarily free sterol. It can be seen in Table I that of the four strains of *A. bisporus* used in this study only strain 310 had a similar ratio of neutral to polar lipids in the fruiting bodies. Free sterol was, however, the predominant lipid in the sporophore neutral fraction.

Utilization of lipid by *A. bisporus* has been shown to be a key factor in the nutrition of the commercial mushroom. Schisler (3) showed that lipid supplementation to compost resulted in increased sporophore yield. Wardle and Schisler (4) showed that lipid additions to basal media gave increased mycelial growth. Ethyl esters of oleic and linoleic acids were shown to be primary stimulatory components.

It is interesting to speculate on the implications of some of the differences in lipid composition of the mycelium and sporophore as found in this study on the lipids of *A. bisporus*. Distinct differences in the weight per cent of the neutral and polar lipids between the sporophore and the mycelium were shown (Table I). The absence of sterol in the mycelium and its appearance in quantity in the sporophore suggests that free sterol is the end product of synthetic events during the fruiting process.

The fatty acids of the sporophore extracts from the various strains were consistent in profile, with the exception of 314 which was significantly lower in linoleic acid than the other three strains. This low level of linoleic acid was concomitant with the fact that strain 314 was giving a low crop yield at the time of sampling. Wardle and Schisler (4) also reported that the growth of mycelium of strain 314 was not increased by ethyl-linoleate supplement. The inability of this strain to utilize linoleic acid may be a cause for low yield. Minor differences in fatty acid composition between strains found in this study are probably attributable to the differences in the metabolism of each strain.

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Chemical Reactions at Lipid-Gas Interfaces: I. Terminal Chain Elongation of Fatty Acids

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ABSTRACT

Reactions of free methyl radicals and monolayers of potassium palmitate and of potassium *n*-heptadecanoate on an aqueous surface yielded straight chain saturated fatty acids containing longer hydrocarbon chains than those of the reactant acids. Similar results were obtained from the reactions of free ethyl radicals and potassium palmitate under similar conditions. The results of this investigation indicate that extension of hydrocarbon chains by free radical addition reactions can be achieved if the growing chains are suitably packed in a monolayer on a surface.

INTRODUCTION

Fatty acids from natural sources are usually predominantly straight chained (1). Extension of a hydrocarbon chain by the random addition of free methyl radicals would be expected to yield a complex mixture of compounds, with straight chain isomers constituting a small fraction of the product (2). However, Wilson (3) has postulated that it would be possible to produce normal paraffins by free radical addition reactions if branching were prevented by crowding the growing chains on a surface so that the reactions could take place only at the terminal methyl groups. This mechanism was postulated as being the method of production of the straight chain fatty acids and *n*-alkane chains found in some meteorites.

In an earlier brief communication (4) we reported the results of a preliminary investigation of Wilson's chain extension mechanism by the reaction of free methyl radicals and monolayers of palmitic acid molecules (as the potassium salt) on an aqueous surface. Under the conditions used in this investigation, the formation of saturated straight chain acids was shown to take place to the exclusion of branched chain isomers. Formation of these straight chain compounds could only have taken place by ω-addition of the methyl radicals to the palmitate. The present paper is a report of the experimental details of our earlier communication, together with further results indicating that similar reactions can be made to occur between methyl radicals and potassium *n*-heptadecanoate and between ethyl radicals and potassium palmitate.

EXPERIMENTAL PROCEDURES

Materials

Di-*t*-butyl peroxide was prepared by the method of Milas and Surgenor (5). Commercial grade lead tetraethyl was distilled under reduced pressure. The distillate collected between 66 and 68 C at 5.5 mm Hg was shown to be pure by gas chromatography.

Chromatographic Analysis

Thin layer (Reverse Phase). Chromatoplates (20 x 20 cm) were covered with a slurry of Silica Gel G (65 g) in distilled water (130 ml) using a Desaga applicator set at 0.75 mm. With this quantity of silica gel, four plates could be prepared for each application of the slurry. The plates were air-dried overnight and activated at 120 C for 2 hr. They were impregnated by a single development with 5% (v/v) solution of undecane in petroleum ether (bp 50-60 C).

Gas-liquid. Lead tetraethyl was analyzed with a Pye Argon gas chromatograph equipped with a Lovelock Ra D ionization detector and glass columns (1.2 m x 3.0 mm) containing the following liquid phases on Celite (80-120 mesh): Apiezon "L" grease (10% w/w) and, Polyethylene glycol 400 (5% w/w).

The columns were operated at 100 C and 50 C respectively with an argon flow-rate of 60 ml/min in both cases.

Methyl esters of fatty acids were analyzed on the first column at 200 C. Another glass column (2.4 m x 6.5 mm) containing Celite (30-80 mesh) coated with polyethylene glycol adipate polyester (20% w/w) at 185 C contained in a laboratory-constructed gas chromatograph (6) was used for identification of the esters.

Identification of the esters was based on agreement of their relative retention volumes (V_R) with those of known compounds when

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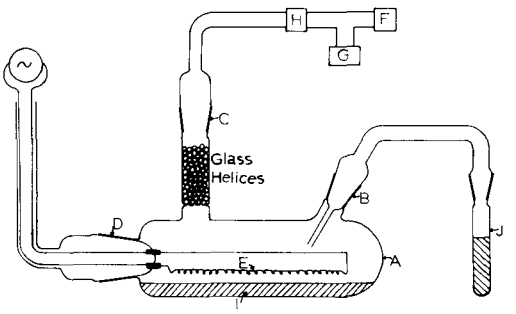

FIG. 1. Reaction apparatus. See text for lettering.

chromatographed on both the polar and non-polar columns. Percentages of the components in the reaction products with hydrocarbon chains of greater length than those of the reactants were calculated by measuring the areas under the gas chromatographic curves and comparing them with that obtained with a known weight of methyl stearate chromatographed under the same conditions.

Reaction Apparatus and Methods

The apparatus used for the study of the free radical addition reactions consisted of a horizontal glass tube (Fig. 1,A) with two 14/35 ground glass sockets for the entry (B) and exit (C) of reactant gases, and a 34/45 socket (D) into which a tungsten filament (E) was fitted. The fatty acid (ca. 2 mg) was dissolved in hot 0.1 N potassium hydroxide solution (50 ml) containing sufficient potassium chloride so that the resulting solution was saturated with the chloride at the temperature at which the reaction was to be studied. When the solution had cooled to room temperature, it was poured carefully into the horizontal tube and the various parts of the apparatus were assembled. All joints were lightly covered to half of their lengths with Apiezon M grease. The apparatus was placed in a low temperature ethylene glycol-water bath that was thermostated at either O C or -10 C. When the apparatus had cooled to the desired temperature, the pressure in the apparatus was reduced to 2-3 mm Hg, as measured on a Vacustat (C), by a mechanical oil vacuum pump (F) and power to the filament (E) turned on.

Methyl radicals were generated by the thermal decomposition of di-*t*-butyl peroxide (5,7) near the heated filament (E) which was suspended approximately 5 mm above the solution (I). Ethyl radicals were produced by a similar method from lead tetraethyl (8). The radical source (6 ml) distilled under reduced pressure from the tube (J) into the vicinity of the heated filament. The voltage (ca. 15 volts)

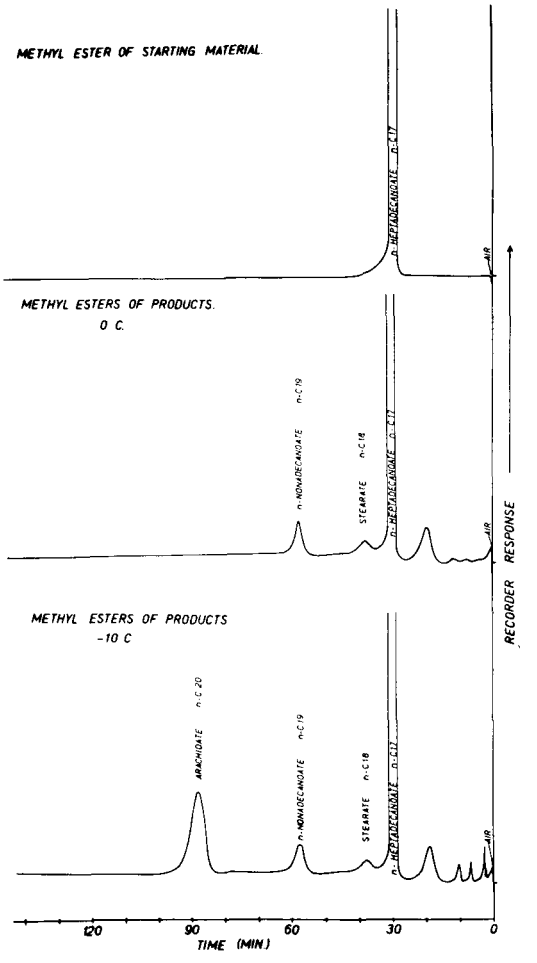

FIG. 2. Gas chromatograms of methyl esters of starting material (*n*-heptadecanoic acid) and products from reactions with methyl radicals at 0 C and -10 C. Apiezon L column (see Experimental Procedures for operating conditions).

across the filament was controlled by a variac so that the temperature of the filament remained at approximately 400 C. In this way decomposition of the radical sources took place in the vicinity of, rather than on, the filament. Water vapour, volatile reaction products and unreacted radical source, which were pumped out of the reaction vessel, condensed in the liquid air trap (H) connected between the reaction vessel and the vacuum pump.

When all the radical source had distilled from the tube (J) (8 hr for the di-*t*-butyl peroxide and 32 hr for the lead tetraethyl), the fatty acid reaction mixture was acidified with 2 N sulfuric acid and extracted with petroleum ether (bp 50-60 C). The fatty acids were esterified with diazomethane and analyzed by gas chromatography.

TABLE I

Yields of Reaction Products[a]

Radical	Acid	Bath Temp., C	Reaction products					
			n-C17	*n*-C18	*n*-C19	*n*-C20	*n*-C21	*n*-C22
Methyl	*n*-C16	0	0.9	2.7				
Methyl	*n*-C16	-10	0.7	1.4	3.5			
Ethyl	*n*-C16	0		0.4		5.5		
Ethyl	*n*-C16	-10		0.5		1.0		11.7
Methyl	*n*-C17	0		0.8	3.1			
Methyl	*n*-C17	-10		0.6	1.8	4.7		

[a]The results are expressed as a weight per cent of the total petroleum ether soluble material of the reaction products after acidification and subsequent esterification. Four runs were completed on each system and the figures quoted are an average of the results from these, the variation between runs being ± 10%. This variation resulted from the inability to exactly duplicate the conditions of each run.

The reaction of methyl radicals and palmitate at -10 C, as described above, was repeated ten times and the resulting mixtures of fatty acids were combined. The mixture of acids (ca. 20 mg) was applied as a streak to four impregnated thin layer chromatoplates. To facilitate location of the required compounds, palmitic, stearic and arachidic acids were applied to a spot at either end of the streak. The chromatograms were developed with acetic acid-water (96:4 v/v). After the solvent and the undecane had been removed in a stream of warm air the plates were sprayed with a 0.2% alcoholic solution of 2',7'-dichlorofluorescein. Compounds corresponding to the positions of stearic and *n*-nonadecanoic acids, located by viewing the plates under UV light, were extracted from the silica gel with acetone. Each extract was applied as a streak to two other impregnated chromatoplates and the separation repeated twice. The purified fatty acids were converted to their methyl esters. Samples of the methyl esters were analyzed with a Perkin-Elmer model 137E IR spectrophotometer and with an AEI model MS9 high resolution mass spectrometer.

RESULTS

The temperature range in which the reactions could be studied was limited to between 0 C and -10 C. Below about -10 C the reaction solutions froze and above 0 C the water evaporated very rapidly. Although a saturated solution of sodium chloride was found to freeze below -20 C, very little sodium palmitate was retained in the saturated sodium chloride solution below 0 C. No reaction was observed when mixtures of ethylene glycol-water were used as the aqueous phase instead of inorganic salt solutions.

Increasing the distance of the heated filament from the aqueous surface resulted in a decrease in the rate of the chain extension reactions because of competing reactions, e.g., methyl radical addition to yield ethane (5). This distance, which was kept at a minimum, was such that the filament, which elongated (sagged) slightly on being heated, was as close as possible to the surface. The radical sources were therefore decomposed as close to the aqueous surfaces as was possible without heat from the filament disturbing the surfaces.

Gas chromatograms of the products, as methyl esters, from the reactions of free methyl radicals and potassium *n*-heptadecanoate at 0 C and -10 C are shown in Figure 2. Similar results were obtained from the reactions of methyl radicals and potassium palmitate (4) and from the reactions of ethyl radicals and potassium palmitate. Unidentified compounds of lesser chain length than the reactants, together with compounds of greater chain length, were present in the reaction products. Insignificant quantities of branched chain or unsaturated acids containing either more than 16 carbon atoms from reactions involving palmitate or more than 17 carbon atoms from reactions involving *n*-heptadecanoate were found in the reaction products.

When methyl radicals and potassium palmitate were reacted on an aqueous surface at 0 C, stearate was the main addition product of the reactions. The only other addition product from this reaction was *n*-heptadecanoate. Under similar conditions at -10 C *n*-nonadecanoate was the main addition product, along with lesser quantities of stearate and *n*-heptadecanoate. Similar results were obtained from the reactions of methyl radicals and potassium *n*-heptadecanoate under the same condition,

i.e., the main addition product from the reactions at 0 C was *n*-nonadecanoate and, at -10 C, arachidate. When ethyl radicals and potassium palmitate were reacted at 0 C the main addition product was arachidate and, at -10 C, behenate. Lignocerate was present in the chromatogram from a run in which ethyl radicals and potassium palmitate were reacted, when the temperature of the cold bath dropped below -10 C.

Quantitative analyses of the reaction products are shown in Table I. Pure palmitic acid was recovered by acidification of the reaction mixture from a control run (reaction of methyl radicals and potassium palmitate) using the procedures described above but with no radical source, indicating that no reactions took place under these conditions.

IR spectra of methyl esters of the fatty acids isolated from the reaction products of methyl radicals and potassium palmitate at -10 C and those of methyl stearate were similar. Mass spectra of the esters were similar to the spectra of compounds of known structure (8).

DISCUSSION

The thermal decomposition of di-*t*-butyl peroxide and of lead tetraethyl have been shown to yield, initially, free radicals (5,7):

$$[(CH_3)_3CO]_2 \rightarrow 2\,(CH_3)_3\,CO^{\bullet} \rightarrow 2\,(CH_3)_2\,CO + 2\,CH_3^{\bullet} \quad [1]$$

$$Pb\,(CH_3\,CH_2)_4 \rightarrow Pb + 4\,CH_3\,CH_2^{\bullet} \quad [2]$$

Free radicals so formed may combine with each other or react with a hydrogen donor (A - H);

$$CH_3^{\bullet} + A - H \rightarrow CH_4 + A^{\bullet} \quad [3]$$

$$CH_3^{\bullet} + A^{\bullet} \rightarrow CH_3A \quad [4]$$

The hydrogen donor may be a parent molecule of the free radicals. In the present investigation the hydrogen donor is the hydrocarbon chain of a fatty acid. If reactions of types 3 and 4 were allowed to take place at random along the hydrocarbon chain of a fatty acid, the reaction products would be expected to consist of a mixture of methyl-branched chain acids. Reactions at the terminal methyl group, that would result in chain extension, would take place only if the methylene groups were protected from the free radicals.

The carboxyl group of a fatty acid, being a polar group, is hydrophillic, whereas the hydrocarbon chain, which is nonpolar, is hydrophobic. Thus, fatty acid molecules on an aqueous surface orient themselves in such a way that the carboxyl groups are dissolved in the water while the hydrocarbon chains project up out of the aqueous surface (9-11). Attractive forces between long chain fatty acid molecules prevent the separation of the molecules and thus these forces contribute to the ability of the molecules to form compact monomolecular films on aqueous surfaces (12). Thus the actual surface of the liquid in contact with the air may be considered to consist of the terminal methyl groups of the fatty acid molecules on the aqueous surface. Attack by free methyl radicals on this surface would result exclusively in reactions taking place at the terminal methyl groups of the fatty acids unless the radicals could penetrate between the hydrocarbon chains.

Results of the experiments described above support the hypothesis describing the structure of fatty acid films on aqueous surfaces and the reactions of free methyl and ethyl radicals with these films. Lack of methyl-branching in the products indicates that, for the compounds investigated, the surface layers of fatty acid molecules were so well packed that the free methyl and ethyl radicals could not penetrate between the hydrocarbon chains of the fatty acids. Under the experimental conditions used, cross-linking of the growing chains by the addition of two free radicals on adjacent chains was minimized by the low concentration of radicals at the surface. Increasing the concentration of free radicals on the surface may result in a more rapid formation of long chain products than was observed during the present investigation, but the possibility of the formation of cross-linked products would also increase. These products were not detected with the analytical techniques that were used.

An unusual feature of the results was that as the reaction temperature was lowered, the chain length of the main reaction product increased. This temperature dependence of the extent of the reactions is probably related to the average time that a fatty acid molecule (or ion) stays on the aqueous surface. As the temperature of the aqueous solution was decreased, the rate of exchange of fatty acid molecules on the surface with those within the solution would be expected to decrease. This variation in the rate of exchange of molecules at the surface and those in solution would result in an increase of the average time that an acid molecule stayed on the surface as the temperature was decreased, thus increasing probability of multiple reactions. The change in solubility of the initial reaction products may also affect the extent of the reactions.

The temperature range in which the reactions could be studied was limited by the

rate of evaporation of the water from the solutions and by the freezing points of the solutions. Use of sodium salts of the fatty acids or a water-soluble organic solvent in the aqueous phases to extend the lower limit of the temperature range was unsuccessful. Addition of ethylene glycol to the aqueous fatty acid solutions probably increases the rate of exchange of fatty acid molecules on the surface with those in solution. Increases in the rate and extent of free radical addition to fatty acids, under the conditions that were used in the experiments reported in this paper, were favored by decreasing the rate of interchange of reactant molecules in solution with those in the monolayers as a consequence of lowering the reaction temperature and the use of inorganic salts, rather than an organic solvent, to effect solubilization of the fatty acids. Increased yields of products at the lower temperatures probably result from slower distillation of the radical sources under these conditions. Results from the control run show that products from the termal decomposition of the free radical sources were essential for the chain extension reactions to take place.

Production of very long chain fatty acids was impractical with the apparatus and experimental conditions that were used in the investigations described in this paper. Use of extended reaction time or immobilized surface of their salts may have resulted in the formation of more long-chained compounds than was observed. The use of *n*-propyl or other higher molecular weight radicals for the chain extension of fatty acid molecules was not studied because of the known instability of those radicals under the experimental conditions used in this investigation (13).

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Chemical Reactions at Lipid-Gas Interfaces: II. Insolubilizing Reactions Induced by an Electrical Discharge

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ABSTRACT

Reactions caused by corona discharges (passing through atmospheres of methane, hydrogen or argon) striking the surfaces of aqueous solutions containing long chain fatty acid salts resulted in the formation of solid and insoluble products. These results show that the fatty acid ions were oriented on the aqueous surfaces in such a way that the hydrocarbon chains projected out of the surfaces. Surface layers of long chain alcohols reacted similarly but short and branched chain compounds were not polymerized under the experimental conditions that were used. When methane or hydrogen was used as the discharge gas, reactions occurred along the hydrocarbon chains of the compounds so that the products consisted of networks of many short hydrocarbon chains. These reactions were probably caused by the penetration of hydrogen atoms into the surface layers. Joining of terminal methyl groups of the hydrocarbon chains was the main reaction observed when argon was used as the discharge gas.

INTRODUCTION

Solid insoluble materials have been produced by the passage of high voltage electrical discharges through gaseous mixtures of methane (or ethane), ammonia and water (1,2) and of methane, ammonia, hydrogen sulfide and water (3). These materials were shown to contain highly cross-linked polyethylene-like structures (3). An unusual feature of these reactions was the absence of liquid products with properties similar to those of the solids. The condensation of small hydrocarbon molecules and the release of hydrogen, with the resultant formation of higher molecular weight gaseous, liquid and solid products, have been shown to take place when gaseous paraffins alone were subjected to various electrical discharges (4).

Wilson (3) has proposed that the initial polymerization reactions that took place when water was present in the discharges resulted in the formation of surface active compounds that were cross-linked, by the discharges, on the aqueous surfaces. The present paper describes a study of the electrical discharge-induced insolubilization of known surface active compounds by a corona discharge.

EXPERIMENTAL PROCEDURES

Potassium chloride solutions (0.1 M, 100 ml) containing the surface active compounds were prepared as follows: (a) A commercial sodium alkyl aryl sulfonate (10 mg), with an aliphatic hydrocarbon chainlength of about 12 carbon atoms, was dissolved in the KCl solution. (b) *n*-Valeric acid (10 mg), palmitic acid (10 mg) or stearic acid (10 mg) was dissolved in a solution (1 ml) containing an excess of potassium hydroxide and added to the KCl solution. (c) *n*-Heptylamine (10 mg) was dissolved in dilute sulfuric acid (1 ml) and added to the KCl solution. (d) Cetyl alcohol (1 mg) was placed on the surface of the KCl solution (in the discharge flask) and allowed to spread over the aqueous surface.

A discharge flask, shown diagrammatically in Figure 1, consisted of a 5 liter flask with a gas inlet and two tungsten wires sealed through the wall of the flask as shown. The arrangement of the tungsten wires minimized conduction of electricity along the surface of the glass. Potassium chloride solution containing a surface active compound was carefully poured into the discharge flask so that it covered the lower tungsten wire. The surface of the solution acted as the lower electrode in the discharge flask, the upper electrode being the second tungsten wire.

After evacuating the discharge flask with a mechanical oil pump via a liquid air trap and maintaining the vacuum for five min to remove traces of air, the discharge gas was introduced to a pressure of 750 mm Hg at 15 C. Electrical power for the discharge was supplied by a 40,000 volt, 50 cycle transformer. Variations in the voltage were made by a "variac" in the primary circuit of the transformer.

Each solution was exposed to the discharge (approximately 15,000 volts) for a minimum period of five days. Any insoluble material that

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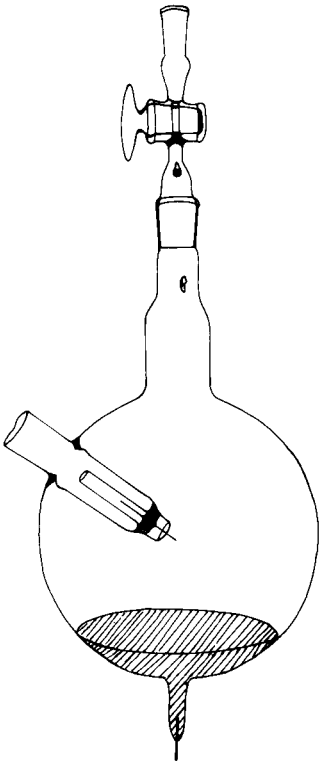

FIG. 1. Diagram of corona discharge flask.

formed on the aqueous surface was removed from the flask, washed with dilute sulfuric acid then with distilled water, until all the salts were removed, and finally with absolute alcohol and dried in a vacuum desiccator. IR spectra of the materials in potassium bromide discs were recorded on a Perkin Elmer model 211 spectrophotometer.

RESULTS

Exposure of aqueous solutions of potassium stearate to corona discharges through either methane, hydrogen or argon resulted in the formation of white insoluble material over all the surfaces in the discharge flask that were exposed to the discharges. The polymer, contaminated with small pieces of tungsten in all instances, was readily dislodged from the walls of the flask by shaking its contents. When newly formed, the polymer had a fine sheet-like appearance, folding readily and breaking into small pieces when the aqueous surface was disturbed. In the form of a surface layer, the polymer was almost transparent, but when dried and in bulk, it had a light yellowish colour, decomposed without melting at 270 C and readily took up dyes, e.g., basic magenta in

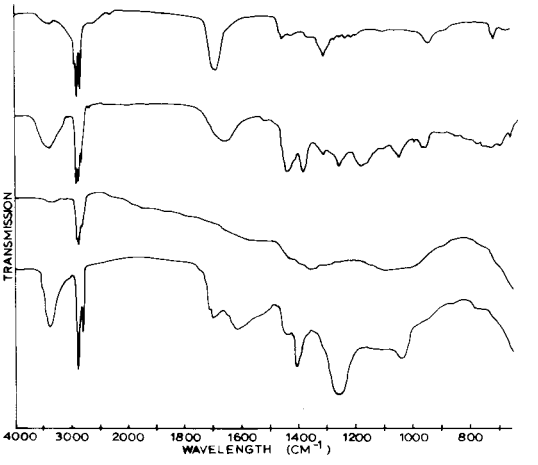

FIG. 2. IR spectra of (from top) stearic acid, polymerized stearic acid using methane as the discharge gas, chromic acid oxidation product of this polymer and polymerized stearic acid using argon as the discharge gas.

water. When dried the polymer was not easily "wet" again by either water or organic solvents and was insoluble in methyl and ethyl alcohol, acetone, glacial acetic acid, ethyl acetate, dimethyl formamide, diethyl ether, petroleum ether (bp 50-60 C), benzene, chloroform and carbon tetrachloride and was unaffected by boiling sodium hydroxide (5 N) or sulfuric acid (5 N) solutions.

IR spectra of the products are shown in Figure 2. By comparing the absorptions at 2950 cm^{-1} (CH_3) and 2912 cm^{-1} (CH_2) of the polymer with those of known compounds (5) (e.g., *n*-hexane and *n*-valeric acid) the hydrocarbon chains in the polymer in which methane was used as the discharge gas was found to be very short (three to four methylene groups per methyl group) as compared with that of the original acid. Stronger absorption, at 3420 cm^{-1}, than would be expected for the hydroxyl group of a carboxylic acid indicated that alcoholic groups were also present in the polymer. Oxidation of the polymer by refluxing it (10 mg) with a solution of sulfuric acid (50% w/v, 10 ml) containing potassium dichromate (50 mg) for 5 hr produced little change in the IR absorption of the product at 2950 cm^{-1} and 2912 cm^{-1} as compared to the original polymer (Fig. 2). Major differences in the spectrum resulting from the oxidation were a decrease in the absorption at 3420 cm^{-1} and general obliteration of the structure below 1800 cm^{-1}.

The passage of an electrical discharge through methane over aqueous salt solutions resulted in the formation of insoluble material on the aqueous surface (6), though at a slower

rate than when potassium stearate was present in the salt solution. To eliminate the possibility that the polymers in the present investigation were being formed by the condensation of methane, hydrogen and argon were used as discharge gases.

Polymers from reactions in which hydrogen was used as the discharge gas were similar in all respects to those in which methane was used. The IR spectrum of the stearate polymer from the reaction in which argon was used as the discharge gas (Fig. 2) showed no methyl absorption at 2950 cm^{-1} and the methylene absorption at 2912 cm^{-1} was very strong, indicating that long unbranched hydrocarbon chains existed in this polymer. Strong absorption at 3420 cm^{-1} indicated that alcoholic groups were also present.

Insoluble polymers which were similar in all respects to those from reactions involving potassium stearate were recovered from corona discharge reactions over solutions of potassium palmitate using atmospheres of methane, hydrogen or argon. Polymers with physical properties similar to those of the fatty acid polymers were also recovered from reactions involving surface layers of cetyl alcohol. No water insoluble polymers were formed on the aqueous surfaces of solutions containing potassium *n*-valerate, *n*-heptylamine sulfate or the sodium alkyl aryl sulfonate when these were exposed to the corona discharge through either hydrogen or argon.

DISCUSSION

The present study has shown that monomolecular layers of long chain fatty acid salts and of long chain alcohols on aqueous surfaces may be insolubilized by a corona discharge. Orientation of the salts or alcohols on the surface must be such that the hydrocarbon chains of the compounds project out of the surface to allow their reaction with the discharge or free radicals in the gas phase. The essential properties required of the compounds in the monomolecular layers to allow insolubilization of the compounds to take place under the corona discharge are that the terminal methyl groups of the long chain compounds are close enough in the monomolecular layers (i.e., the layers have a close-packed structure) to allow them to join and that the compounds remain in the layer for a sufficient time to allow the reactions to take place.

Insolubilization of short chain compounds was probably prevented by both the high rate of exchange of molecules of the compounds in the solution with those on the surface and the relatively high solubility of the reaction products. Attractive forces between the hydrocarbon chains of short chain compounds are insufficient to allow these compounds to form compact surface monolayers and the organic molecules in the surface may be gaseous. The surface active compound having an aromatic ring in its structure (sodium alkyl aryl sulfonate) did not form a polymer when solutions of it were exposed to the corona discharge because either the hydrocarbon chains on the aqueous surface were too far apart (although the surface area of the sulfonate in a monolayer is only slightly larger than that of a long chain fatty acid) or because of the high rate of exchange of molecules in the monolayer with those in solution.

Exposure of surface layers of fatty acid salts to the corona discharge through methane did not result in chain extension of the fatty acids as was observed when they were reacted with free methyl radicals (6-8). In the present study, the concentration of free radicals in the gas phase near the aqueous surface was probably high enough to allow the cross-linking reactions to occur at the expense of reactions leading to the addition of methyl radicals to the monolayers.

All attempts to prevent contamination of the polymers with tungsten were unsuccessful, even when the electrodes were cleaned between experiments. This contamination did not interfere with examinations of the physical properties of the polymers but prevented their detailed elemental analysis.

Joining of terminal methyl groups of the fatty acids was found to be the predominant reaction taking place on the aqueous surface when argon was used as the discharge gas. The short chain lengths of the polymers when methane or hydrogen was used as the discharge gas can be explained by the following reaction mechanisms: (a) The discharge, on passing through the surface layer, caused cross-linking to occur along the chains, but this would not explain the absence of cross-linking along the chains when argon was used. (b) When methane was used as the discharge gas, the polymer was gradually built up from a heavily cross-linked portion.(c) Hydrogen atoms from the primary reactions;

$$CH_4 + e^- \text{ (fast)} \rightarrow CH_3^{\bullet} + H^{\bullet} + e^- \text{ (slow) and}$$
$$H_2 + e^- \text{ (fast)} \rightarrow 2H^{\bullet} + e^- \text{ (slow)}$$

were able to penetrate between the hydrocarbon chains and cause cross-linking along the chains. Previous investigations showed that free methyl radicals cannot penetrate between the hydrocarbon chains of compounds under experimental conditions similar to those that were used in the present project (6-8).

The results presented above suggest that it is possible to use an electrical discharge to produce very large flat macromolecules of a specific orientation by insolubilizing monomolecular layers of sufficiently long chain compounds on an aqueous surface. Small pieces of the polymers may be expected to fold in such a way as to form microspheres (micelles) with the carboxyl groups being directed towards the centres of the spheres. Small spheres were observed in samples of the reaction solutions containing potassium palmitate or potassium stearate when they were viewed under an optical microscope. On the basis of other investigations (6), it is suggested that short chain compounds may also be cross-linked to form solvent insoluble polymers if the temperature of the reaction were lowered to such an extent that the time a molecule stayed on the surface was sufficient to allow extensive multiple additions to occur.

ACKNOWLEDGMENT

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Characterization of Branched Monounsaturated Hydrocarbons of *Sarcina lutea* and *Sarcina flava*

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ABSTRACT

The exact position of the double bond and the probable branch assignments of each hydrocarbon isomer of *Sarcina lutea* and *Sarcina flava* were determined by derivatives and gas chromatography and combined gas chromatography-mass spectrometry. It was shown by mass spectral data that in all isomers the double bond position was in or near the center of the molecule and, that some of the resolved isomers contained an additional positional isomer. The hydrocarbons were identified as containing methyl branches in the iso or anteiso or both configurations, symmetrically and asymmetrically disposed on the ends of the isomers. The assignment of the methyl branches for each tetrad of four isomers as the isomers emerged from the gas chromatographic column was iso-iso'; anteiso-iso; anteiso-anteiso'; and iso-normal for odd carbon-numbered chains, and iso-iso'; anteiso-iso; iso-normal; and anteiso-normal for even carbon-numbered chains. A fifth isomer was identified as a normal-olefin.

INTRODUCTION

Studies on the identification of aliphatic hydrocarbons of various members in the family *Micrococcaceae* have revealed tetrads of methyl-branched acyclic olefins for each carbon fraction in the range C22-C30 (1,2). Each tetrad is composed of four isomers, most of which have methyl groups in either the iso or anteiso configuration, both symmetrically and asymmetrically disposed on the ends of the molecules (1-3). One of the hydrocarbon components in the C29 fraction of *Sarcina lutea* FD 533 has been identified as having both iso and anteiso branch methyl configurations on opposite ends of the molecule (3,4). The exact assignment of branching and double bond position to the isomers in each carbon fraction has not yet been reported.

Albro and Dittmer (3) have reported that the double bond position in the hydrocarbons of *S. lutea* is near the center of the molecule (3). Their information was obtained by periodate-permanganate oxidation of hydrocarbon fractions. The present report contains evidence for identifying the exact position of the double bond in each hydrocarbon isomer of *S. lutea* and *Sarcina flava* as well as probable branching assignments. The identifications were based on results obtained from derivatives and gas chromatography and combined gas chromatography-mass spectrometry.

MATERIALS AND METHODS

Unsaturated Hydrocarbons

Sarcina lutea strains ATCC 533 (1,2) and FD 533 (2,3) and *Sarcina flava* ATCC 540 (2) were grown in trypticase soy broth (BBL) to the early stationary phase of growth. The cells were harvested by centrifugation and washed twice with saline solution. The methods used to extract the cells and to fractionate the lipids on silicic acid columns have been previously described (2). The hydrocarbons were eluted from the columns with *n*-hexane.

Procedure for Preparing Trimethylsilyl Ether Derivatives of Hydroxylated Hydrocarbons

The olefinic hydrocarbons were routinely oxidized to diols with osmium tetroxide (5). The hydrocarbons (ca. 1 mg) were suspended in 1 ml dioxane and 0.1 ml of dioxane containing 1 mg OsO_4 was added. The mixture was placed in a water bath for 1 hr at 37 C. The mixture was acidified with 2.5% methanolic-HCl (made by passing HCl gas through methanol) and then made alkaline with a few drops of 7 N NaOH (NaCl crystals appear but do not interfere). The mixture was placed in a 37 C water bath for 1 hr. A sufficient quantity of water (ca. 9/10 of the mixture volume) was added to form an aqueous phase and the hydroxylated hydrocarbons extracted with ethyl ether. After the solvent was removed under a stream of N_2, 0.5 ml of bis-trimethylsilyl acetamide (BSA) was added. The mixture was evaporated to dryness in vacuo and the trimethylsilyl ether (TMSE) derivatives redissolved in $CHCl_3$ prior to analyses. Close to 100% of the hydrocarbons were recovered as TMSE-derivatives.

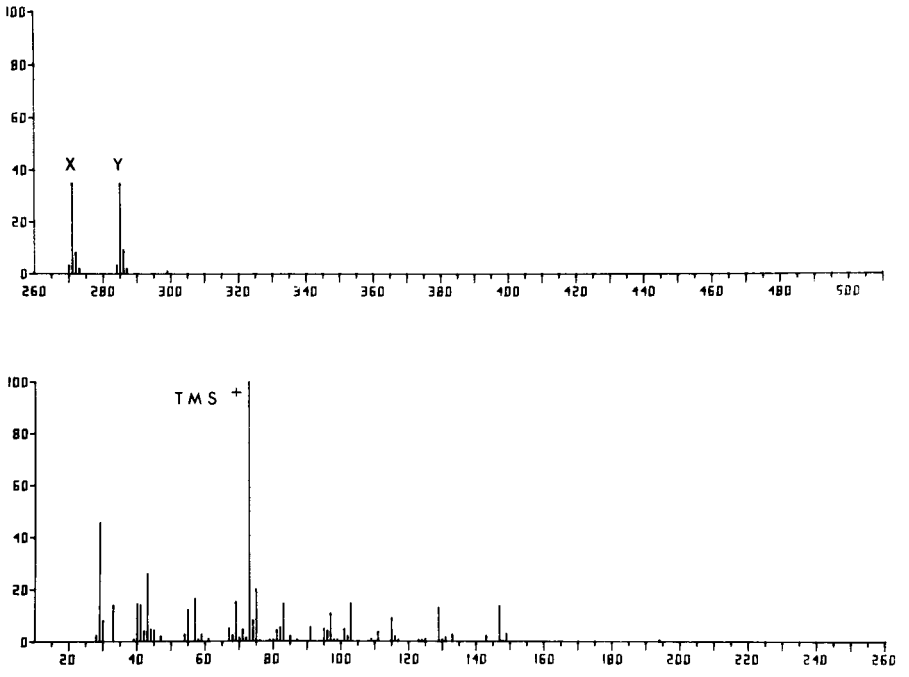


FIG. 1. Mass spectrum of the trimethylsilyl ether derivative of an oxidized monounsaturated C27 hydrocarbon.

The procedure of Wolff et al. (6) was initially employed in this study for preparing TMSE-derivatives of hydroxylated hydrocarbons. The procedure was modified by washing the Na_2SO_3 sediment with ethyl ether to recover the higher molecular weight hydroxylated hydrocarbons. The ether extract was added to the original supernatant fraction and treated as described.

Gas Chromatography and Mass Spectrometry

The aliphatic hydrocarbons and the TMSE-derivatives of hydroxylated hydrocarbons were analyzed in an F & M 5750 Gas Chromatograph equipped with dual flame ionization detectors and an AEI MS-12 Mass Spectrometer connected to a Beckman GC 45 Gas Chromatograph (7). The chromatographic analyses were carried out in 62 m x 0.05 cm stainless steel capillary columns coated with OV-17 (methyl phenyl silicone) or with Apiezon L (a high temperature grease).

Mass spectra were taken as each component emerged from the column into the ion source of the mass spectrometer. Mass spectra were recorded repetitively from m/e 500-28 at 10 sec intervals using a small computer data system. Temperatures of the connecting lines, helium separator and ion source were maintained at 270-300 C. The ionizing potential was 70 eV; ionizing current was 100 μa.

Permanganate-periodate Oxidation

Hydrocarbon fractions, isolated by preparative gas chromatography, were converted to fatty acids by the oxidation of the double bonds according to the permanganate-periodate procedure described by Albro and Dittmer (3). The fatty acids were identified by comparing their chromatographic retention times to those obtained for *S. lutea* fatty acids that were previously identified by GLC-MS analyses (1,2). The chromatographic analyses were carried out in a 91 m x 0.076 cm stainless steel column coated with Igepal CO 990 (nonyl phenoxy polyoxyethylene ethyl alcohol) under conditions previously described (1,2).

RESULTS AND DISCUSSION

Identification of Double Bond Position in Hydrocarbon Components

Mass spectra of the TMSE-derivatives of oxidized monounsaturated hydrocarbons have been interpreted in a manner analogous to that described for TMSE-derivatives of oxidized monounsaturated fatty acid esters (8). A complete mass spectrum obtained as one component emerged from the gas chromatograph is shown in Figure 1. The spectrum is dominated by the $(CH_3)Si+$ ion at m/e 73. Prominent peaks at m/e 271 and 285 of nearly equal intensity are the only structurally significant

TABLE I

Trimethylsilyl Ether Derivatives of Hydroxylated Hydrocarbons of *Sarcina lutea* and *Sarcina flava*

Derivatives	Peak no.[a]	*S. lutea* ATCC 533			*S. flava* ATCC 540		
		Ret. time[b], min	Moles %[c]	Carbon fragments, x:y[d]	Ret. time[e], min	Moles %	Carbon fragments, x:y[d]
Br-C23-TMSE[f]	1	25.3	2.60	11:12	---	---	---
	2	26.0	1.80	11:12	---	---	---
	3	26.7	0.67	---	---	---	---
	4	27.4	0.33	---	---	---	---
Br-C24-TMSE	5	30.5	5.51	12:12	---	---	---
	6	31.3	5.15	12:12[g]; 11:13	---	---	---
	7	32.7	1.20	12:12[g]; 11:13	---	---	---
	8	33.5	2.10	11:13	---	---	---
Br-C25-TMSE	9	35.7	6.01	12:13	37.1	0.08	---
	10	36.5	20.75	12:13	37.6	0.10	---
	11	37.3	12.63	12:13	38.4	0.49	---
	12	38.0	0.54	---	39.2	0.12	---
Br-C26-TMSE	13	40.2	2.21	13:13[g]; 12:14	41.6	1.46	13:13[g]; 12:14
	14	41.0	7.15	12:14[g]; 13:13	42.2	1.43	13:13[g]; 12:14
	15	42.2	1.00	13:13	43.8	0.12	---
	16	43.0	4.48	13:13	44.6	0.14	---
Br-C27-TMSE	17	45.0	1.14	13:14	47.4	0.83	13:14
	18	46.5	8.12	13:14	48.2	16.48	12:15
	19	47.7	16.61	13:14	49.1	5.89	12:15
	20	---	---	---	50.0	3.50	13:14
	a	---	---	---	53.1	8.89	13:14
Br-C28-TMSE	21	---	---	---	54.1	2.78	13:15
	22	---	---	---	55.2	4.98	14:14
	23	---	---	---	58.1	5.36	13:15[g]; 14:14
	24	---	---	---	59.4	13.35	13:15[g]; 14:14
Br-C29-TMSE	25	---	---	---	64.0	0.41	14:15
	26	---	---	---	65.9	4.99	14:15
	27	---	---	---	67.7	18.35	14:15
	28	---	---	---	69.5	2.05	14:15
	b	---	---	---	76.7	2.76	14:15
Br-C30-TMSE	29	---	---	---	78.7	0.42	---
	30	---	---	---	80.9	0.37	---
	31	---	---	---	86.7	1.39	15:15
	32	---	---	---	89.3	3.29	15:15

[a]The peak numbers correspond to the peak numbers of the hydrocarbons previously presented (2).

[b]Gas chromatographic separation of the derivatives was obtained on a 62 m x 0.05 cm stainless steel capillary column coated with 5% OV-17; helium flow rate ca. 15 ml/min; temperature programmed from 200 to 270 C in 64 min.

[c]Mole per cent compositions of the hydrocarbons were calculated on the basis of their gas chromatographic areas, which were obtained by multiplying the peak heights by the widths at half peak heights.

[d]Letters x and y represent the carbon number of each component obtained after cleavage of the TMSE-derivative by mass spectroscopy.

[e]Gas chromatographic separation of the derivatives was obtained on a 62 m x 0.05 cm stainless steel capillary column coated with 10% Apiezon; helium flow rate ca. 10 ml/min; temperature programmed from 170 to 250 C in 40 min.

[f]Symbols: Br, branching; TMSE, trimethylsilyl ether.

TABLE II

Relative Percentages of the Compositions of C27, C28 and C29 Hydrocarbons of *Sarcina Lutea* FD 533[a]

Proposed configuration	Per cent Hydrocarbons		
	C27	C28	C29
Iso-iso'	12.4	15.1	20.6
Anteiso-iso	32.8	22.5	39.6
Anteiso-anteiso'	44.3	---	37.4
Iso-normal	6.9	38.0	2.4
Anteiso-normal	---	24.3	---
Normal (peak a)	3.6	---	---

[a]The hydrocarbon composition has been previously presented by Tornabene et al. (2). The retention time values of the components are identical to those presented for the same carbon fractions of *S. flava* ATCC 540 in Table I.

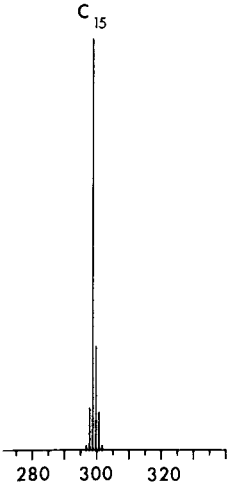
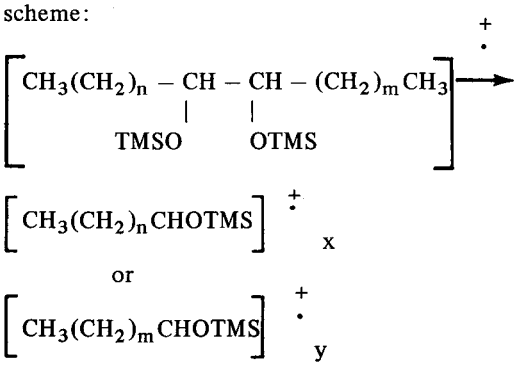


FIG. 2. Partial mass spectrum showing the dominant peak representing trimethylsilyl ether fragments of an even carbon-numbered chain (C30).

ions observed. Unlike the mass spectra of TMSE derivatives of oxidized monounsaturated fatty acid esters, there are no detectable ions at higher m/e values (M+ or M-15 ions). From the retention times and characteristic chromatographic pattern (Table I), the component yielding the mass spectrum in Figure 1 was expected to result from a C27 parent olefin. Interpretation of this mass spectrum confirms this assignment. The ions at m/e 271 and 285 correspond to the fragments x and y when n=11 and m=12 in the general fragmentation scheme:



Localization of the positive charge on either OTMS group accounts for the equal probability of observing fragments x or y. The large alkyl residue does not influence the relative intensities of the two principal ion products because the branching sites are seven or more carbons distant from the charge bearing atoms.

In a similar manner, mass spectral data for each component permitted determination of double bond position. Ions x and y were found to be of nearly equal intensity when n does not equal m, and a single major ion is produced when n equals m. A partial mass spectrum for a C30 component is shown in Figure 2. The major peak at m/e 299 indicates the parent olefin had a centrally positioned double bond. The fragment ions x and y were thus used to position the double bond and indicate the molecular weight and carbon number of each component. Molecular weight assignments of every component examined were confirmed by GLC-MS analyses of saturated alkanes resulting from hydrogenation of the olefins. No attempts were made to identify the double bond positions in compounds present in small amounts.

A summary of the results obtained for two of the organisms studied is given in Table I. Detailed data from the analysis of *S. lutea* FD 533 hydrocarbons are not presented because of its equivalence to the data obtained on the hydrocarbons of *S. flava*. Since the unsaturated position in the isomers does not play a role in the chromatographic profile (1) of the aliphatic hydrocarbons, the distribution patterns of the TMSE-derivatives and the aliphatic hydrocarbons (1,2) are identical. Therefore, the peak numbers assigned correspond to the peak numbers of the hydrocarbons previously described (2).

It is apparent from Table I that the hydrocarbon compositions consist of multiple isomers that are readily separated by gas chromatography. It was shown by mass spectral data that the isomers have the same carbon number and that the double bond position is near the center of the molecule; and, that some of the isomers, separated by GLC as symmetrically shaped peaks, contain an additional

TABLE III

Per Cent Distribution of the Structures of the Aliphatic Terminae of the C27, C28 and C29 Hydrocarbons of *Sarcina Lutea* FD 533

Branch Configuration	Per cent distribution[a]			Expected per cent distribution[b]		
	C27	C28	C29	C27	C28	C29
Iso	34.8	45.0	41.5	32.25	45.35	41.60
Anteiso	57.8	25.2	57.3	60.70	23.40	57.20
Normal	7.3	29.8	1.1	7.05	31.15	1.20

[a]Determined from the fatty acids produced by periodate-permanganate oxidation of the double bonds.

[b]Determined by calculating the percentages of the branch configurations that would be obtained if the hydrocarbon components presented in Table II, containing the branching configurations assigned, were fragmented at the double bonds.

positional isomer. The occurrence of unresolved mixed positional isomers has been observed only in even carbon-numbered chains (Table I). Although the relative percentages of the mixed positional isomers were not calculated, a letter g was placed alongside the carbon fragments in Table I to indicate which positional isomer was predominant. Resolved positional isomers are present in the C24 fraction of *S. lutea* ATCC 533 and the C27 and C28 fractions of *S. flava*. On the other hand, the position of the double bond in all five resolved isomers of the C29 fraction of *S. flava* is between carbons 14 and 15.

The fragmentation of the TMSE-derivatives of the molecules in the ranges C23 - C27 and C25 - C30 resulted in two moieties that were different only by a maximum of three carbon atoms in any one hydrocarbon composition. These results are consistent with the different modes of entry of a few selected fatty acid molecules proposed by Albro and Dittmer (9-11) for the head to head biosynthesis of hydrocarbons in *S. lutea*.

The hydrocarbon composition of *S. lutea* FD 533, comparable to that of *S. flava* except for the relative per cent proportion of the individual components, has been presented before in considerable detail (2,3). Mass spectra have shown that the positions of the double bond in the hydrocarbons of *S. lutea* FD 533 are also the same as those described above for *S. flava*. The only major difference between the hydrocarbon compositions (2) of *S. lutea* ATCC 533 and those of *S. flava* and *S. lutea* FD 533 is in the range of the hydrocarbons. The nature of the components and the positions of the double bond are basically the same. Since this exact type of hydrocarbon pattern has been found to be distributed among members of the family *Micrococcaceae*, the positional isomers reported here may also be representative of the hydrocarbons of other *Sarcina* and *Micrococcus* species (2).

Assignment of Methyl Branches

The identities assigned to the individual aliphatic hydrocarbon isomers by Tornabene et al. (1,2) as olefins containing iso or anteiso methyl branching on either one or both ends of the molecules cannot explain the difference in the chromatographic separation of the isomers found in the hydrocarbon fractions of even and odd carbon-numbered chains. Previously, methyl branching assignments were made on the basis of mass spectral data indicating the following character for the emerging tetrad patterns: even numbered chains were iso, anteiso, iso, anteiso; odd carbon-numbered chains were iso, anteiso, anteiso, iso. Differences in retention times were assumed to be due to symmetrically and asymmetrically disposed branches (1,2; Table I). Evidence for the occurrence of iso and anteiso methyl branches on opposite terminae of the chains, however, has been reported for one of the hydrocarbon components in the C29 fraction of *S. lutea* FD 553 by Albro and Dittmer (3) creating a discrepancy in the identification of the C29 components in the two reports (2,3). Unambiguous assignment of the exact position of the methyl branches from the present mass spectral data could only be possible by comparing the data to that of authentic standards (12). However, from the retention time values and identification of the oxidation products of the hydrocarbons, together with the previously presented mass spectral data (1), it is possible to assign identities to the isomers in each tetrad.

The relative retention times of the isomers in the even carbon-numbered fractions are different from those in the odd carbon-numbered fractions (Table I). The difference is seen in the chromatographic retention times of the last two components in each group; the four isomers in the even carbon-numbered fractions are divided into dyads while the four isomers of the odd carbon-numbered fractions are more closely

grouped together (the differences between the two groups are exemplified in the chromatographic profiles previously presented in References 1 and 2). The relative chromatographic retention time values of the isomers in each carbon group reveal that the third isomer in both the even and odd carbon-numbered chains are not the same structural components. In fact, the relative position of the fourth isomer in the odd carbon-numbered fractions is comparable to the position of the third isomer in the even carbon-numbered fractions. It appears that three of the isomers are common to both even and odd carbon-numbered chains. On the basis of the relative retention time values of the isomers and eluting sequences of components containing methyl branches in the iso and anteiso configurations, the branching configurations of the isomers were proposed to be for odd carbon-numbered chains iso-iso', anteiso-iso, anteiso-anteiso', and iso-normal; and, for even carbon-numbered chains iso-iso', anteiso-iso, iso-normal and anteiso-normal. The time differentials between the iso-iso' and anteiso-iso components and between the anteiso-iso and iso-normal components are comparable in both even and odd carbon-numbered chains (Table I). The identifications assigned to the components are still in general accordance with the branching configurations previously assigned by mass spectral analyses of these components (1), with the exception of the second component in each tetrad being a molecule containing both iso and anteiso methyl branches on opposite ends of the chain rather than the anteiso configuration previously assigned to it (1,2).

Components designated as peaks a and b (Table I) have been identified by their molecular weights, determined by GLC-MS, as the fifth isomers in the C27 and C29 fractions of the hydrocarbons of *S. flava*. These components were tentatively identified as normal-olefins. The retention time values support these assignments. These two peaks were not identified in the hydrocarbon compositions previously reported (2). The fifth isomers have only been found in substantial quantities in a few of the organisms studied (2).

Evidence supporting the proposed identities of the components was obtained from comparing the relative per cent composition of the individual components to the relative per cent composition of the fatty acids produced by periodate-permanganate oxidation of the double bond of the hydrocarbons in isolated C27, C28 and C29 fractions. The proposed identities and per cent composition of the major hydrocarbon fractions of a representative organism, *S. lutea* FD 533 (2), are presented in Table II. The per cent distribution of the branch configurations in the C27, C28 and C29 hydrocarbons is presented in Table III. The most probable configurations of the individual hydrocarbons that can be calculated from the percentage composition of the hydrocarbons and the percentage distribution of the branch configurations are the branching assignments presented in Table II.

The fact that only odd carbon-numbered fatty acids contain anteiso methyl branches (1) together with the proposed hydrocarbon biosynthetic mechanism of head to head condensation of two fatty acids, with one molecule of fatty acid undergoing decarboxylation (9-11), is fully consistent with the proposal that anteiso-anteiso' branched hydrocarbons occur only in the odd carbon-numbered tetrads.

ACKNOWLEDGMENT

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Role of the Bases and Phosphoryl Bases of Phospholipids in the Autoxidation of Methyl Linoleate Emulsions[1]

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ABSTRACT

Methyl linoleate, emulsified in borate buffer with sodium lauryl sulfate, was used to study the pro- or antioxidant effect of 0-phosphocholine, 0-phosphoethanolamine, 0-phosphoserine, as well as the corresponding nonphosphoryl compounds. Oxygen uptake was calculated from rate data obtained at 37 C with an oxygen electrode. The results were similar for the corresponding phosphoryl and nonphosphoryl bases. 0-Phosphocholine and choline had little effect at either pH 7.9 or 10.2. 0-Phosphoethanolamine and ethanolamine significantly increased oxygen uptake at pH 7.9, but significantly decreased uptake at pH 10.2. 0-Phosphoserine and serine decreased oxygen uptake at both pH values. The catalytic activities of the bases investigated may be attributed to their functional groups. The phosphoryl and β-hydroxy groups exhibited no catalytic activity in the autoxidation of methyl linoleate emulsions at either pH 7.9 or 10.2. The α-carboxyl amino group of 0-phosphoserine and serine decelerated autoxidation at both pH values. The amino group $H_3\overset{+}{N}$ of the primary amine accelerated autoxidation, but the H_2N: group had the reverse effect. Since the quaternary amino group $(CH_3)_3\overset{+}{N}$ did not affect autoxidation at either pH 7.9 or 10.2, we concluded that the presence of the N-H bond may be necessary for the prooxidant activity of an amine, and that the presence of a pair of free electrons on the nitrogen of an amine is necessary for its antioxidant activity. Kinetically, the autoxidation of methyl linoleate emulsion without added base was in agreement with Farmer's proposed mechanism involving a bimolecular dissociation of hydroperoxides. However, methyl linoleate emulsion at pH 7.9 and 37 C in the presence of ethanolamine or 0-phosphoethanolamine was autoxidized by a mechanism involving a combined mono- and bimolecular dissociation of hydroperoxides.

INTRODUCTION

The unsaturated fatty acids that occur in lipids are susceptible to undesirable changes caused by autoxidation. It is generally recognized that the unsaturated acids are autoxidized primarily by a free radical chain reaction as first postulated by Farmer et al. (1-3). New free radical initiations are largely derived from product hydroperoxides via a monomolecular and/or a bimolecular decomposition.

Autoxidation of phospholipids is far more complicated than in the case of the neutral triglycerides. The bases or phosphorylated bases of phospholipids have long been suspected to affect the autoxidation of the unsaturated fatty

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TABLE I

Coefficients a_0, a_1 and a_2 of Equation 1, Which Represents Rate of Oxygen Uptake as a Function of Time

Base added	a_0		a_1		a_2	
	pH 7.9	pH 10.2	pH 7.9	pH 10.2	pH 7.9	pH 10.2
Control	3.0599	1.5184	0.1478	0.3890	0.0064	0.0105
Choline	2.4406	1.4091	0.1308	0.3336	0.0051	0.0087
Ethanolamine	3.5303	0.5124	0.7448	-0.0799	-0.0004	0.0259
Serine	1.9928	0[a]	-0.1226	0[a]	0.0041	0[a]
O-Phosphocholine	3.3837	2.1044	0.2700	0.5307	0.0055	0.0097
O-Phosphoethanolamine	3.4160	0.4511	0.6008	0.0264	-0.0038	0.0018
O-Phosphoserine	1.9052	0[a]	0.0345	0[a]	0.0071	0[a]

[a]No oxygen absorption observed up to 33 hr.

acids present (4). The bases may "remove" free radicals or product hydroperoxides from the system and act thereby as antioxidants, or they may decompose product hydroperoxides to new radicals and thus act as prooxidants. Some studies of the effect of phosphoryl bases on autoxidation of homogeneous oil systems have been reported previously (5).

The purpose of this study was to explore the effects of functional groups of the phosphoryl bases of phospholipids on the autoxidation of unsaturated lipids in aqueous emulsion. Methyl linoleate emulsions were used in the presence and absence of various phosphoryl bases. 0-Phosphocholine, 0-phosphoethanolamine and 0-phosphoserine, which are the phosphoryl bases of phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine, respectively, were selected for this study. These compounds were selected not only because they are the phosphoryl bases of the glycerol phospholipids most widely distributed in nature, but also because of the similarity of their chemical structures. In addition, the catalytic effects of choline, ethanolamine and serine on the autoxidation of methyl linoleate emulsion were investigated.

MATERIALS AND METHODS

Materials

Materials used were boric acid, sodium borate and sodium hydroxide (Baker Chemicals); sodium lauryl sulfate (Sigma); methyl linoleate (chromatographically pure), 0-phosphoserine, serine, 0-phosphoethanolamine, ethanolamine hydrochloride, 0-phosphocholine in the form of 0-phosphocholine calcium chloride, and choline in the form of chloride salt (Calbiochem). Each was used without further purification except 0-phosphocholine calcium chloride. Since calcium ion would precipitate in borate buffer, this compound was converted to its sodium salt by passage through a Dowex 50 ion exchange column.

Aqueous solutions of each of the above compounds were analyzed for copper and iron contamination with a Perkin-Elmer Atomic Absorption Spectrophotometer, Model 303. Negative results were obtained for all compounds except serine, which was contaminated with 0.06 ppm copper, and 0-phosphoserine, which was contaminated with 0.05 ppm copper and 0.11 ppm iron. Attempts to remove the contaminants by recrystallization and by elution through a Dowex 50 column were unsuccessful.

Each original 1 g vial of methyl linoleate was fractionated into five Pasteur pipets. These

TABLE II

Catalytic Activity of Bases as Related to Predominant Structure in Presence of Methyl Linoleate Emulsions

pH	Choline	Ethanolamine	Serine	pH	O-Phosphocholine	O-Phosphoethanolamine	O-Phosphoserine
7.9	$HOCH_2CH_2\overset{+}{N}(CH_3)_3$[a]	$HOCH_2CH_2\overset{+}{N}H_3$[b]	$HOCH_2CH(COO^-)\overset{+}{N}H_3$[c]	7.9	$^-O-\overset{O}{\overset{\uparrow}{P}}-OCH_2CH_2\overset{+}{N}(CH_3)_3$[a] (O$^-$)	$^-O-\overset{O}{\overset{\uparrow}{P}}-OCH_2CH_2\overset{+}{N}H_3$[b] (O$^-$)	$^-O-\overset{O}{\overset{\uparrow}{P}}-O-CH_2CH(COO^-)\overset{+}{N}H_3$[c] (O$^-$)
10.2	$HOCH_2CH_2\overset{+}{N}(CH_3)_3$[a]	$HOCH_2CH_2\ddot{N}H_2$[c]	$HOCH_2CH(COO^-)\ddot{N}H_2$[d]	10.2	$^-O-\overset{O}{\overset{\uparrow}{P}}-OCH_2CH_2\overset{+}{N}(CH_3)_3$[a] (O$^-$)	$^-O-\overset{O}{\overset{\uparrow}{P}}-OCH_2CH_2\ddot{N}H_2$[c] (O$^-$)	$^-O-\overset{O}{\overset{\uparrow}{P}}-OCH_2CH(COO^-)\ddot{N}H_2$[d] (O$^-$)

[a]Practically no catalytic activity.
[b]Prooxidant.
[c]Antioxidant.
[d]Strong antioxidant.

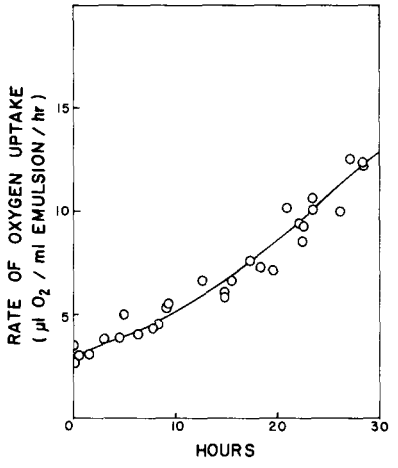

FIG. 1. Rate of oxygen uptake by reference methyl linoleate emulsion at pH 7.9. The circles represent experimental data. The best fit curve was calculated from the corresponding equation in Table I.

were sealed on both ends with a torch after flushing with nitrogen, and were then stored at -20 C until used. The methyl linoleate had an average peroxide value of 3.4 ± 0.6 mEq/kg as determined by the method of Hills and Thiel (6), and the value did not change with storage time.

Apparatus

A Biological Oxygen Monitor Model 53 (Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio) was used to measure oxygen absorption by methyl linoleate emulsions. The apparatus consists of two modified Clark-type oxygen electrodes, an electronic amplifier, a bath stirrer assembly with four reaction vessels, and a constant temperature circulator (Lauda Model K-2). The reaction vessels hold 3 to 8 ml of solution stirred synchronously by a teflon-coated stirring bar in each. The electrodes are fitted into two lucite plungers whose outside diameter closely matches the inside diameter of the reaction vessels. It is assumed that a closed system is obtained when a plunger is inserted into a reaction vessel.

A thin (0.001 in.) teflon membrane isolates the anode and cathode from the experimental solution but allows diffusion of oxygen into the electrode. When a known polarizing current is applied across the cell, oxygen reacts at the cathode, causing a current to flow through the cell at a rate proportional to the amount of oxygen diffusing across the membrane. The diffusion of oxygen is directly proportional to the pressure differential and a linear relationship exists between the external oxygen pressure and the cell current.

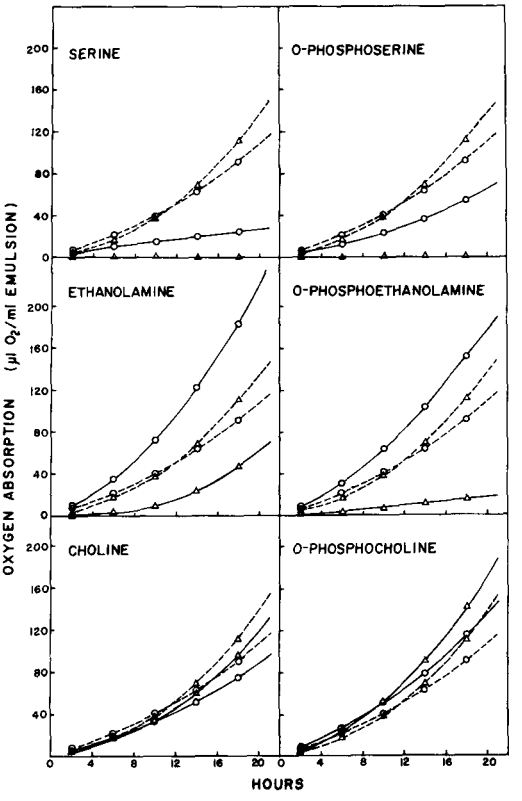

FIG. 2. Amounts of oxygen absorbed at pH 7.9 and 10.2 by methyl linoleate emulsions containing O-phosphocholine, choline, O-phosphoethanolamine, ethanolamine, O-phosphoserine or serine. O-- Control at pH 7.9; O— base added at pH 7.9; Δ-- control at pH 10.2; Δ— base added at pH 10.2.

The change in oxygen tension can be read directly from the meter on the amplifier. However, in our study, the signal was fed continuously from the electrode to the amplifier and to a 100 mv recorder (Varian Aerograph Model 20) which was operated at a speed compatible with the rate of oxygen uptake. Thus the rate of oxygen uptake by the emulsion could be calculated from the slope of recorded curve, provided that the electrode was calibrated with water under identical conditions.

Preparation of Emulsions

Approximately 0.50 M solutions of O-phosphocholine, O-phosphoethanolamine, O-phosphoserine, choline, ethanolamine and serine were prepared with water. The pH value of one portion of each solution was adjusted to 7.9 and the other portion to 10.2 with sodium hydroxide. Then, each base solution was diluted to a concentration of 0.21 M.

Boric acid-borate buffer (0.10 M, pH 7.9)

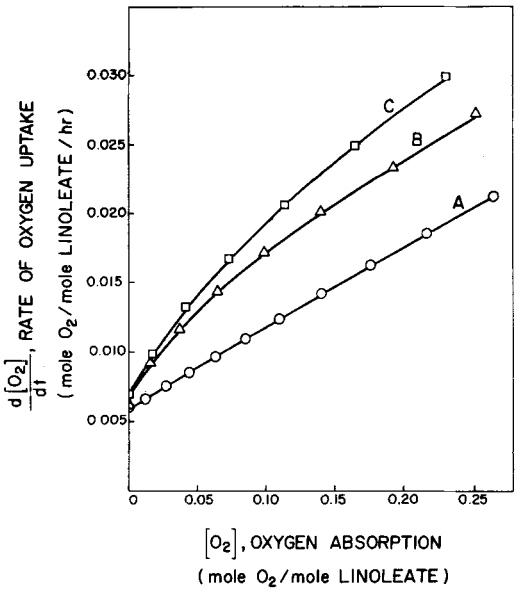

FIG. 3. Rate of autoxidation of methyl linoleate emulsions in relation to extent of oxygen absorption. A; Reference methyl linoleate emulsion containing 0.02 M methyl linoleate and 0.002 M sodium lauryl sulfate in 0.1 M borate buffer at pH 7.9. B; Methyl linoleate emulsion containing compounds in A plus 0.01 M 0-phosphoethanolamine. C; Methyl linoleate emulsion containing compounds in A plus 0.01 M ethanolamine.

and borate-sodium hydroxide buffer (0.10 M, pH 10.2) were prepared according to Dawson et al. (7). Sodium lauryl sulfate solutions (0.002 M) were prepared from each of the two borate buffer solutions.

The lauryl sulfate buffer solutions at each pH were emulsified with methyl linoleate to give emulsions of known composition. Emulsification was done in a 25 ml widemouth glass vial with a Sonifier Cell Disruptor (Model W-185D, Heat Systems Co., Melville, N.Y.). The power output of the Cell Disruptor was 60 to 65 watts, and a 0.5 in. tip was used. The mixture was emulsified for 30 sec and then cooled in an ice bath for 1 min. Emulsification was repeated twice more. The resulting emulsion was stable for at least four days at room temperature.

Immediately after emulsification, 4.0 ml of emulsion and 0.2 ml of one of the 0.21 M base solutions at the same pH were mixed in a reaction vessel of the Biological Oxygen Monitor. Water (0.2 ml) was mixed with 4.0 ml of emulsion as a control. The concentration of each compound in the final emulsions was the following: methyl linoleate, 0.02 M; base, 0.01 M; borate, 0.095 M; and sodium lauryl sulfate, 0.002 M.

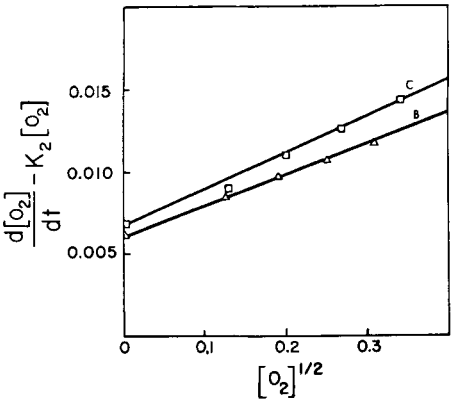

FIG. 4. A plot of $d[0_2]/dt\text{-}K_2[0_2]$ versus $[0_2]^{1/2}$ for methyl linoleate emulsions containing ethanolamine (C) or 0-phosphoethanolamine (B). Composition of emulsions was the same as in Figure 3.

Calculation of Rate of Oxygen Uptake by Methyl Linoleate Emulsions

The Oxygen Monitor was calibrated at atmospheric pressure in water saturated with air at 37 C. Emulsions were constantly stirred at 37 C in contact with air in the reaction vessels. The oxygen tension of each emulsion was measured over a suitable interval, beginning 15 min after the base solution was mixed into the emulsion. The rate of oxygen uptake was calculated from the slope of the curve of the recorded oxygen tension. The relation between rate of oxygen uptake and oxidation time was expressed with a quadratic equation:

$$d[O_2]/dt = a_0 + a_1 t + a_2 t^2 \qquad [1]$$

where $d[0_2]/dt$ = rate of oxygen uptake by emulsion in μl 0_2/ml emulsion/hour and t = oxidation time in hours. The coefficients a_0, a_1 and a_2 were calculated for each emulsion from the experimental data according to the method of least squares (8). The generation of three normal equations from the experimental data for a least squares fit to the quadratic equation, and the solution of these equations to obtain constants a_0, a_1 and a_2 were carried out with an Olivetti-Underwood Programa 101 Desk Computer. The computer programs used were obtained from the library of Olivetti-Underwood.

Calculation of Amount of Oxygen Absorbed by Methyl Linoleate Emulsions

The amount of oxygen absorbed by each emulsion was calculated by integration of the corresponding rate equation with respect to time.

$$[O_2] = \int d[O_2]\,dt \cdot dt = C + a_0 t + a_1 t^2/2 + a_2 t^3/3$$

$$[O_2] = b_1 t + b_2 t^2 + b_3 t^3 \qquad [2]$$

where $b_1 = a_0$; $b_2 = a_1/2$; $b_3 = a_2/3$. The integration constant C was evaluated to be zero with the initial condition that no oxygen was absorbed by the emulsion at the starting instant.

RESULTS

Reproducibility of Method

The reproducibility of the method was examined by following six replicate runs of oxygen uptake by methyl linoleate emulsion at pH 7.9. When the best fit curve was obtained for these data (Fig. 1), the deviation of individual experimental results from the curve did not exceed 10%.

Effect of Various Bases on Oxygen Absorption

Values of the constants a_0, a_1, and a_2 of Equation I, which represents the rate of oxygen uptake by various methyl linoleate emulsions as a function of time, are listed in Table I.

The amounts of oxygen absorbed by the various emulsions as a function of time are plotted in Figure 2. At pH 7.9, ethanolamine significantly increased oxygen absorption as compared to the control. In contrast, oxygen absorption was slightly decreased by the addition of choline and significantly decreased by the addition of serine. At the same pH, 0-phosphoethanolamine significantly increased absorption, while absorption was only slightly increased by addition of 0-phosphocholine and appreciably decreased by the addition of 0-phosphoserine.

The effects of the bases at pH 10.2 were as follows: Oxygen absorption by methyl linoleate emulsion was slightly decreased by the addition of choline. Addition of ethanolamine significantly decreased oxygen absorption, while addition of serine practically completely inhibited oxidation for at least 22 hr. 0-Phosphocholine slightly increased absorption, while addition of 0-phosphoethanolamine significantly decreased absorption. Addition of 0-phosphoserine resulted in practically complete inhibition for at least 22 hr.

Effect of pH on Oxygen Absorption

Effects of pH on oxygen absorption by methyl linoleate emulsions in the presence and absence of added base are also shown in Figure 2. An increase of pH from 7.9 to 10.2 did not significantly change absorption by the control emulsions. Also, the curves for oxygen absorption by methyl linoleate emulsions containing choline or 0-phosphocholine were similar to that of the control emulsions. However, the increase in pH changed both ethanolamine and 0-phosphoethanolamine from prooxidants to antioxidants. The increase in pH increased the antioxidant activity of both serine and 0-phosphoserine.

The chemical structure at pH 7.9 and 10.2 and the pro- or antioxidant activity of the six bases tested are summarized in Table II.

DISCUSSION

The six compounds studied have the same carbon skeleton (Table II). Choline, ethanolamine and serine also have the same β-hydroxy group; but choline is a quaternary amine, ethanolamine a primary amine and serine an α-amino acid. Since each of these compounds contains one or more ionizable groups, their chemical structures in aqueous solutions depend on the pH of the solution. The predominant forms of these compounds in emulsions at pH 7.9 and 10.2 can be predicted, since the dissociation constants of the ionizable groups are known (9).

Effects of Phosphoryl and Hydroxy Groups

Each phosphoryl derivative of choline, ethanolamine and serine has the same catalytic effect as the parent compound when compared at the same pH (Table II). This indicates that the phosphoryl group has the same effect as the hydroxy group it replaced. However, the β-hydroxy group probably does not influence autoxidation of methyl linoleate emulsions. Marcuse (10) found that alanine, serine and glycine had similar antioxidant activity in linoleic acid emulsions. The structures of serine and alanine are identical except that serine has a hydroxy group instead of a hydrogen at the β-carbon position. It is therefore reasonable to conclude that the catalytic activities in the present study are not related to either the β-hydroxy or the phosphoryl group.

Effects of Amino Group

The predominant forms of ethanolamine and 0-phosphoethanolamine at pH 7.9 are different from their corresponding forms at pH 10.2 (Table II). At pH 7.9, the amino group of these amines is positively charged; at pH 10.2 it becomes neutral with a pair of free electrons on the nitrogen, since the pK value of the amino group of ethanolamine is 9.5 (9).

Ethanolamine and 0-phosphoethanolamine increased oxygen uptake at pH 7.9, indicating that the positively charged amino group $H_3\overset{+}{N}$ of

these two amines is capable of accelerating autoxidation of emulsion. In contrast, ethanolamine and 0-phosphoethanolamine at pH 10.2 retarded oxygen uptake, indicating that the H_2N: group of these two amines is capable of decelerating autoxidation. Since choline and 0-phosphocholine, both quaternary amines, did not affect autoxidation of methyl linoleate emulsion at pH 7.9 or 10.2, it may be presumed that the presence of the N-H bond is necessary for the prooxidant activity of an amine, and that the presence of a pair of free electrons on the nitrogen of the amine is necessary for the antioxidant activity.

Effects of α-Carboxyl Amino Group

The antioxidative activity of serine and 0-phosphoserine at both pH 7.9 and 10.2 is evident from Figure 2. The antioxidant activity of both compounds is greater at pH 10.2 than at pH 7.9. Marcuse (10) reported that several amino acids, including serine, have a potential antioxidative effect in methyl linoleate emulsions prepared with phosphate buffer and Tween (emulsifier). He also reported the antioxidative activities of these amino acids were higher at pH 9.5 than at pH 7.5.

In the present study, the change at pH 7.9 from a prooxidant to an antioxidant when a carboxyl group was introduced to the α-carbon of amine is of particular interest. The α-carboxyl amino group has many unique chemical properties that distinctly differ from those of a simple amino or carboxyl group. For example, α-carboxylamines, but not β-carboxylamines, undergo oxidative deamination under light and have synergistic action with phenolic antioxidants (11). Also α-amino acids were oxidized by hydrogen peroxide to α-keto acids but δ- and ε-amino acids were inert to oxidative deamination (12).

The serine used in the present study was contaminated with 0.06 ppm copper, while the 0-phosphoserine had 0.05 ppm copper and 0.11 ppm iron. Because of the chelating ability of serine and 0-phosphoserine, we were unable to eliminate these contaminants. In general, copper and iron at low concentrations function as powerful prooxidants of lipid autoxidation. However, in our study, the chelated metal ions did not accelerate autoxidation of methyl linoleate emulsion (Fig. 2). This may indicate that serine and 0-phosphoserine function as metal deactivators as well as autoxidation inhibitors.

Mechanism of Autoxidation of Methyl Linoleate Emulsion

The rate of oxygen uptake by emulsion containing only methyl linoleate is proportional to the first order of oxygen absorbed as indicated by curve A of Figure 3. The linear relationship indicates that a bimolecular decomposition of hydroperoxides may be involved in the autoxidation (13). Marcuse and Fredrikson (14) reported a similar relationship between the rate of oxygen uptake and its absorption by linoleate emulsions prepared with phosphate buffer and Tween 20 (emulsifier) in the range of pH 5 to 7.

The linear relationship between the rate of oxygen uptake and the amount of oxygen absorbed can be expressed by the equation:

$$d[O_2]/dt = K_2[O_2] + b \qquad [3]$$

where $d[0_2]/dt$ = rate of oxygen uptake by methyl linoleate in mole 0_2/mole linoleate/hr; $[0_2]$ = oxygen absorption by methyl linoleate in mole 0_2/mole linoleate; K_2 = rate constant of bimolecular reaction in $hour^{-1}$; and b = initial rate of autoxidation in mole 0_2/mole linoleate/hr. The rate constant K_2 of the autoxidation of methyl linoleate in emulsion at pH 7.9 and 37 C, calculated from Curve A of Figure 3, is 0.056/hr. This value is in good agreement with the bimolecular rate constant, 0.065/hr at 37 C, reported by Mabrouk and Dugan, Jr. (15). They used phosphate buffer (0.1 M) and starch (emulsifier, 0.25%) to prepare emulsions, and measured oxygen uptake manometrically.

The bimolecular rate constant of the autoxidation of neat ethyl linoleate at 37 C is 0.045/hr, as calculated from the data reported by Bolland and Gee (16). Thus the rate constant of autoxidation of the neat linoleate corresponds closely to that of linoleate in emulsion (0.056/hr) when both are calculated on the basis of unit mole of linoleate. This provides further support for the hypothesis (17) that linoleate either neat or in emulsion is autoxidized according to the same mechanism during the early stages.

Mechanism of Autoxidation of Methyl Linoleate Emulsion With Added Ethanolamine or 0-Phosphoethanolamine at pH 7.9

As indicated in Figure 3, the rate of oxygen uptake by the methyl linoleate emulsions containing ethanolamine or 0-phosphoethanolamine at pH 7.9, is linearly proportional to the first order of oxygen absorption after 0.1 mole of oxygen has been absorbed. This indicates that the autoxidation of methyl linoleate emulsions in the presence of ethanolamine or 0-phosphoethanolamine, takes place by a bimolecular decomposition of hydroperoxides only after 0.1 mole of oxygen has been

absorbed. The bimolecular rate constant K_2 of Equation 3 is also about 0.056/hr, since the linear portions of Curves B and C tend to parallel that of Curve A.

At early stages of the autoxidation, however, the value of $d[O_2]/dt\text{-}K_2[O_2]$ of methyl linoleate emulsions containing ethanolamine or 0-phosphoethanolamine is linearly proportional to the square root of oxygen absorbed (Fig. 4). This linear relationship may be represented by the equation:

$$d[O_2]/dt - K_2[O_2] = K_1 [O_2]^{1/2} + b$$
$$\text{or } d[O_2]/dt = K_2[O_2] + K_1[O_2]^{1/2} + b \qquad [4]$$

where K_1 = rate constant of monomolecular reaction in (mole O_2/mole linoleate)$^{1/2}$/hr.

Equation 4 is a general rate law representing a combination of mono- and bimolecular autoxidations. The straight lines of Figure 4 indicate that, from the kinetic point of view, methyl linoleate is oxidized by a mechanism of combined mono- and bimolecular reactions. The parallelism of the two straight lines confirms the similarity of the reaction mechanism for ethanolamine and its 0-phosphoryl derivative.

Initiation of a significant monomolecular decomposition of hydroperoxides by the addition of ethanolamine or 0-phosphoethanolamine may be explained by the electronegativity of the nitrogen atom of the amines. When ethanolamine or 0-phosphoethanolamine is added to methyl linoleate emulsions at pH 7.9, complexes of primary amine-hydroperoxide may form through hydrogen bonding between hydrogen of the amino group and oxygen of the hydroperoxide ($R'H_2\overset{+}{N}\text{–}H\ldots\overset{H}{O}OR$). Such complexes may decrease the hydroperoxide-hydroperoxide (ROOH.....$\overset{H}{O}$OR) complex formation and may be followed by initiation of the pseudomonomolecular decomposition of hydroperoxide. Bateman and Hughes (18) have reported the modification of order of hydroperoxide decomposition from second to first by the addition of hydrogen bonding compounds in homogeneous systems.

Mechanism of Autoxidation of Methyl Linoleate Emulsions With Added Ethanolamine or 0-Phosphoethanolamine at pH 10.2

Oxygen uptake by methyl linoleate emulsion at pH 10.2 was decreased by addition of ethanolamine or 0-phosphoethanolamine, but no induction period was observed (Fig. 2). This indicates that these amines are not primarily free radical inhibitors, but are hydroperoxide decomposers as previously suggested by Ingold (19). The decomposition reaction probably involves the complexing of amine and hydroperoxide by a hydrogen bond through the pair of free electrons of the nitrogen atom and the hydrogen of the peroxide group ($R_3N\text{:}\ldots HOOR$), followed by reduction of hydroperoxide to the corresponding alcohol through a nonradical pathway. Similar reactions in homogeneous systems have been reported by previous investigators (20,21). The one-to-one adduct of amine and hydroperoxide bonded together by a hydrogen bond through the free electrons of the nitrogen atom and the hydrogen of the peroxide has been demonstrated by IR spectroscopy (22,23).

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Ratio of in Vivo Incorporation of ^{3}H Arachidonic Acid and ^{14}C Linoleic Acid Into Liver Lipids From Normal and Diabetic Rats

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ABSTRACT

Normal and streptozotocin diabetic rats were injected via the portal vein with a labeled solution containing ^{3}H arachidonic acid and ^{14}C linoleic acid ($^{3}H/^{14}C$ ratio, 0.5) during a 1 min period. Livers were quickly frozen, pulverized, and the lipids extracted and fractioned by thin layer chromatography. The incorporation of ^{3}H and ^{14}C into liver lipids was measured and the percentage distribution of radioactivity into the different lipid fractions was determined. The incorporation of ^{14}C linoleic acid and ^{3}H arachidonic acid into liver lipids is apparently reduced in rats with severe diabetes. The higher $^{3}H/^{14}C$ ratio found in the 1,2 diglycerides from diabetic rats may be explained by the apparently smaller incorporation of ^{14}C linoleic acid or by an isotopic dilution attributable to the great availability of this acid in diabetic rats. On the other hand, the higher $^{3}H/^{14}C$ ratio observed in triglycerides and phospholipids from diabetic rats, due to a relatively large incorporation of ^{3}H arachidonic acid into this fraction, may be explained by the affinity of the enzymes involved in their synthesis for some 1,2-diglyceride units. Insulin was unable to correct the changes observed in the diabetic rats.

INTRODUCTION

Studies in several laboratories have established that diabetic rats are unable to desaturate fatty acids at the same rate as normal rats (1-4). This fact accounts for a low synthesis of arachidonic acid in the diabetic rat, demonstrated in vivo by Friedman et al. (5), and the possible role of this defect in the physiopathological disturbances observed in diabetes. Peluffo et al. (6) were able to demonstrate in vivo a depression of the conversion of linoleic acid to arachidonic acid by rat testes with severe diabetic atrophy. Brenner et al. (7) reported that the testicular atrophy developed by rats with severe diabetes could be prevented by arachidonic acid. Since it has been observed previously (8) that arachidonic acid is the principal polyunsaturated fatty acid component of the different lipids isolated from cellular particles, it was of interest to determine the ratio of incorporation of this acid and its pre-

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TABLE I

Incorporation of ^{3}H Arachidonic Acid and ^{14}C Linoleic Acid Into Total Lipids of Livers From Normal, Diabetic and Diabetic Plus Insulin Rats

Experimental group	Counts/min/g liver[a]		Ratio $^{3}H/^{14}C$
	^{3}H Arachidonic acid	^{14}C Linoleic acid	
Normal (5)[c,d]	124,355 ± 64,475[b]	265,820 ± 95,000	0.46
Diabetic (4)	34,370 ± 13,550 $P < 0.05$	60,830 ± 22,250 $P < 0.05$	0.56
Diabetic +	25,930 ± 11,500 $P < 0.05$	47,485 ± 19,250 $P < 0.05$	0.54

[a]The radioactivity is expressed as counts/min/g liver.

[b]Values are the means ± standard deviations of the means.

[c]Probability (P) values are related to normal animals.

[d]Figures in parentheses represent number of animals. Experimental conditions are indicated in Methods.

TABLE II

Ratio of Counts/Min/G Liver From ^{3}H Arachidonic Acid and ^{14}C Linoleic Acid Incorporated Into Lipid Fractions

Experimental group	Ratio $^{3}H/^{14}C$			
	1,2 Diacylglycerol	Triacylglycerol	3-sn-Glycero-phosphorylcholine	3-sn-Glycero-phosphoryl-ethanolamine
Normal (5)[a,b]	0.16 ± 0.03[c]	0.38 ± 0.02	0.78 ± 0.14	1.35 ± 0.13
Diabetic (4)	0.27 ± 0.07 P < 0.05	0.79 ± 0.07 P < 0.001	1.27 ± 0.28 P < 0.01	1.41 ± 0.41 NS[d]
Diabetic + insulin (4)	0.35 ± 0.12 P < 0.05	0.75 ± 0.03 P < 0.001	1.07 ± 0.08 P < 0.001	1.18 ± 0.32 NS

[a]Numbers in parentheses indicate the number of animals in each group.

[b]Date are the means ± standard deviations of the means.

[c]Probability (P) values are related to normal animals.

[d]Not significant.

cursor, linoleic acid, into the lipid components of liver cells from diabetic and normal rats.

EXPERIMENTAL PROCEDURES

Material and Methods

1-^{14}C Linoleic acid (52,9 mC/mmole) was purchased from Radiochemical Center (Amersham, England). It was 98% radiochemically pure and had <2% *cis-trans* unsaturated acid. ^{3}H Arachidonic acid (280 μC/mg) was provided by P. Hoffman, La Roche, Basle, Switzerland. It was >99% radiochemically pure as proved by gas liquid chromatography. Phospholipase-A was obtained from the Ross Allen Institute (USA). Insulin (40 U/ml) was purchased from the Lilly Laboratories of Argentina, and streptozotocin was provided by Upjhon Laboratories (USA). Silica Gel G was purchased from Merck (Germany).

Animals

Male albino Wistar rats weighing 150-170 g and fed a balanced diet were used. Streptozotocin diabetes was induced by intravenous injection of 50 mg of streptozotocin per kilogram of body weight. Only those animals showing a fasting blood glucose level of 300 mg/100 ml or more were used. The animals were fasted during 6 hr prior to the experiment.

Preparation of Injection Solution

1-^{14}C Linoleic acid and ^{3}H arachidonic acid were dissolved in 0.1 ml 0.1 N KOH and the solution was heated at 25 C until a nearly clean soap solution was obtained (9); then 1.5 ml of rat serum was added. After standing overnight in N_2 atmosphere, at room temperature with magnetic stirrer, this solution was used directly. 0.1 ml Contained 10 μC of ^{14}C linoleic acid and 5 μC of ^{3}H arachidonic acid.

Injection of Labeled Solution

A group of five rats was anesthetized with ether and their portal vein exposed. The labeled solution, 0.1 ml per rat, was injected at a fairly constant rate during a 1 min period. Immediately following the injection, the liver was frozen between two aluminum blocks that had been precooled in liquid N_2 and then pulverized in a precooled mortar. The powder was extracted by the method of Folch et al. (10).

One group of five diabetic rats was injected in the same way and another group of four diabetic rats was injected with the labeled solution plus 1 U of insulin per rat. The livers of both groups were processed as described above.

The lipids were recovered from the original chloroform-methanol extract and separated by thin layer chromatography (TLC).

Separation of Lipids

Phospholipids were separated into their components by TLC using chloroform-methanol-water (65:25:4 v/v/v) (11). The spots, identified by comparison with authentic standards and visualized by I_2 vapors, were scraped off, transesterified with methanolic HCl (12), extracted with light petroleum, evaporated and assayed for radioactivity.

Neutral lipids were also separated into their components by TLC in petroleum ether-ethyl ether-acetic acid (80:20:1 v/v/v) (13) and assayed as mentioned above. However, since, in liver extracts, cholesterol stains too deeply to permit direct observation of diglyceride, this whole region was routinely scraped off as one band and saponified with 5% KOH in methanol. Cholesterol was extracted with petroleum

TABLE III

Per Cent of the Total Radioactivity Recovered From the ^{3}H Arachidonic Acid and ^{14}C Linoleic Acid Incorporated Into Lipid Fractions

Experimental group	Per cent distribution of radioactivity							
	1,2 Diacylglycerol		Triacylglycerol		3-sn-Phosphorylcholine		3-sn-Phosphoryl-ethanolamine	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
Normal (5)[a,b]	9.61 ±[c]	26.73 ±	31.80 ±	35.59 ±	33.60 ±	20.44 ±	10.56 ±	4.03 ±
	1.30	3.95	1.43	4.42	6.51	2.68	1.42	0.81
Diabetic (4)	8.75 ±	18.62 ±	27.36 ±	20.14 ±	35.72 ±	17.67 ±	8.32 ±	4.40 ±
	2.08	2.63	8.18	6.37	1.81	1.58	1.40	2.25
	NS[d]	$P < 0.01$	NS	$P < 0.001$	NS	$P < 0.05$	NS	NS
Diabetic + insulin (4)	12.55 ±	21.88 ±	22.41 ±	16.03 ±	38.52 ±	25.44 ±	8.75 ±	3.78 ±
	3.90	3.09	8.32	4.58	13.90	7.85	1.59	2.10
	NS	$P < 0.05$	$P < 0.01$	$P < 0.001$	NS	NS	NS	NS

[a]Numbers in parentheses indicate the number of animals in each group.

[b]Probability (P) values are related to normal animals.

[c]Data are the means ± standard deviations of the means.

[d]NS, not significant.

TABLE IV

Positional Distribution of Radioactive Fatty Acids Incorporated Into 3-sn-Phosphatidylcholine and 3-sn-Phosphatidylethanolamine

Experimental group	Fatty acid	3-sn-Phosphatidylcholine		3-sn-Phosphatidylethanolamine	
		1 Position, %	2 Position, %	1 Position, %	2 Position, %
Normal (5)[a,b]	18:2	7.20 ± 0.25[c]	92.80 ± 0.25	7.20 ± 0.80	92.80 ± 0.80
	20:4	4.00 ± 0.71	96.00 ± 0.71	6.20 ± 2.09	93.80 ± 2.10
Diabetic (4)	18:2	14.00 ± 3.80 P < 0.1	86.00 ± 3.80 P < 0.1	18.25 ± 9.80 P < 0.1	81.75 ± 9.80 P < 0.1
	20:4	4.90 ± 1.15 NS[d]	95.10 ± 1.15 NS	7.50 ± 3.50 NS	92.50 ± 3.30 NS
Diabetic + insulin (4)	18:2	14.00 ± 6.22 P < 0.05	86.00 ± 6.22 P < 0.05	25.33 ± 3.90 P < 0.001	74.67 ± 3.90 P < 0.001
	20:4	5.66 ± 3.03 NS	94.34 ± 3.04 NS	6.33 ± 3.50 NS	93.67 ± 3.50 NS

[a]Numbers in parentheses indicate the number of animals in each group.

[b]Probability (P) values are related to normal animals. See methods in the text.

[c]Data are the means ± standard deviations of the means.

[d]NS, not significant.

ether. The water soluble layer was acidified and the free fatty acids derived from diglycerides which had been extracted with light petroleum, were evaporated and assayed for radioactivity.

Hydrolysis of Phospholipids

Phosphatidylcholine and phosphatidylethanolamine, separated by TLC as described, were extracted from the silica gel using the two phase system of Arvidson (14). After evaporation of the solvent, the phospholipids were dissolved in 5 ml of diethyl ether and 1 mg of *Crotalus adamanteus* venom in 0.2 ml 0.1 M borate buffer pH 7.0, containing 0.04 M calcium acetate, was added. The ether buffer system was shaken vigorously overnight at room temperature. Following the hydrolysis, the mixture was dried under reduced pressure. The dried lipids were dissolved in chloroform-methanol (9:1 v/v) and the free fatty acids and lysoderivative were separated by TLC as described previously, and assayed for radioactivity.

Radioactivity Measurements

Radioactivity measurements were performed in a Packard tri-Carb Scintillation spectrometer, Model 3003. Under the conditions used, ^{3}H showed an efficiency of about 40% and ^{14}C about 53% with a spillover of ^{14}C into the ^{3}H channel of 28%. The ^{14}C channel was free of tritium counts. Tritium activities were corrected for the spillover of ^{14}C.

RESULTS AND DISCUSSION

The capacity of the liver to esterify the injected ^{3}H arachidonic acid and ^{14}C linoleic acids is summarized in Table I.

The total radioactivity incorporated per gram of normal liver is about five times greater than that of the livers from diabetic rats, and insulin was not able to modify this significant change, at least in its early stages. Although the dilution of the injected labeled acids, during their transport in the blood or during their integration into the liver acyl CoA pool, could account for this finding, changes in the activity of enzymes leading to the biosynthesis of phospholipids and neutral lipids cannot be ruled out.

While the $^{3}H/^{14}C$ ratio for the total radioactivity incorporated into liver lipids remains practically constant in the three groups of rats and similar to the corresponding $^{3}H/^{14}C$ ratio of the injected labeled solution, the distribution of radioactivity from ^{3}H arachidonic acid and ^{14}C linoleic acid incorporated into the different lipid fractions leads to quite different $^{3}H/^{14}C$ ratio values (Table II).

As shown in Table II, the $^{3}H/^{14}C$ ratio for the radioactivity incorporated into 1,2-diacylglycerol is significantly higher in the diabetic rats than in the normal rats. This observation, as will be seen later, could be due to a smaller incorporation of ^{14}C linoleic acid in the diabetic groups.

The increase of the $^{3}H/^{14}C$ ratio observed in the triaglycerol fractions suggest either a preferential esterification of the free position of 1,2-diacylglycerol by arachidonic acid or the selective acylation by the enzyme, acyl-CoA-1,2-diacylglycerol-0-transferase in certain diglyceride units.

The $^{3}H/^{14}C$ ratio in the neutral fractions

were significantly higher in the diabetic rats.

Table II also shows the $^{3}H/^{14}C$ ratio for the radioactivity incorporated into the major phospholipid fractions.

According to Kennedy (16), the principal biosynthetic route to 3-sn-phosphatidyl-choline and 3-sn-phosphatidylethanolamine utilized diglyceride as an intermediate. The $^{3}H/^{14}C$ ratio found in normal rats was 0.16 for the diacylglycerol and 0.78 for the phosphatidylcholine. This finding suggests that fatty acid redistribution by the deacylation-reacylation cycle (17) or the relative substrate specificity of the enzyme CDP–choline. 1,2-diglyceride choline phosphotransferase (E.C.1.7.8.2) (18) could be responsible for the difference.

The $^{3}H/^{14}C$ ratio in the 3-sn-glycerophosphorylcholine fraction was significantly higher in the diabetic rats.

Table III summarized the relative distribution of the total radioactivity, expressed as percentage of ^{3}H arachidonic acid and ^{14}C linoleic acid incorporated into the principal lipid fractions.

It may be noted that the similar distribution of ^{3}H counts in 1,2-diacylglycerol from normal, diabetic and diabetic plus insulin rats, coincident with a relatively smaller incorporation of ^{14}C counts into this fraction from diabetic rats, can account for the higher $^{3}H/^{14}C$ ratio found in 1,2-diacylglycerol from these rats (Table II).

The observed increase of the relative incorporation of arachidonic acid into triglycerides from the three groups of rats can explain the increase in the $^{3}H/^{14}C$ ratio from diglycerides to triglycerides (Table II).

As is shown in Table III, the relatively higher percentage of ^{3}H arachidonic acid than ^{14}C linoleic acid incorporated into 3-sn-phosphatidylcholine and 3-sn-phosphatidylethanolamine, can explain the highest $^{3}H/^{14}C$ ratio observed in all the lipid fractions studied and corroborates the results given in Table II.

Table IV summarizes the positional distribution of ^{3}H arachidonic acid and ^{14}C linoleic acid in 3-sn-phosphatidylcholine and 3-sn-phosphatidylethanolamine.

It is interesting to note the increased amount of ^{14}C linoleic acid incorporated into the 1 position of the phospholipid fractions from diabetic and diabetic plus insulin rats compared with control rats (19).

On the other hand, no significant differences were observed on the positional distribution of arachidonic acid in phospholipids from diabetic rats as compared with the normal rats.

In conclusion, we can say that the incorporation of linoleic acid and arachidonic acid into liver lipids is apparently reduced in rats with severe diabetes. The relatively higher incorporation of ^{14}C linoleic acid into the 1 position of phospholipid moieties could be attributed to the high rate of exchange of this acid in the diabetic rats, and this fact could also explain, by an isotopic dilution, the apparent relatively smaller incorporation of this acid into 1,2 diglycerides. The relatively higher incorporation of ^{3}H arachidonic acid into triglycerides and phospholipids could be explained through the affinity of the enzymes involved in their synthesis for certain diglyceride units. Under the conditions of this experiment, insulin was unable to correct the changes observed in the diabetic rats. Studies on the fatty acid composition of the acyl-CoA pool from normal and diabetic rat liver, aimed at resolving these questions, are currently in progress.

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Mono- and Polyenoic Acid Distribution in Plasma Nonesterified Fatty Acids in Kwashiorkor

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ABSTRACT

Plasma NEFA levels were determined in Kwashiorkor (17 cases) and normal infants (13 cases) by Dole's method. The distribution of mono- and polyenoic acids was investigated by Abdel-Wahab's method. Plasma NEFA levels were found to be significantly lower in Kwashiorkor infants (445 μEq/L ± 32) than in normals (591 μEq/L ± 66). Plasma oleic, linoleic and linolenic acids were detected in measurable amounts in both Kwashiorkor and normals, but were significantly higher in Kwashiorkor. Arachidonic acid was hardly detectable in either group. The high levels of mono- and polyenoic acids in Kwashiorkor can be attributed to demands on them for the synthesis of fat and/or preferential utilization of the unsaturated fatty acids in mobilization of other lipid components.

INTRODUCTION

A diet leading to Kwashiorkor is known to be rich in carbohydrate, low in fat and poor in protein. Such a diet was reported to produce low levels of blood lipids (1,2). This hypolipidemia together with the intense accumulation of fat in the liver and abundance of fat in the depots suggest the presence of a defect in lipid metabolism affecting its transport from depots and liver to the blood stream. Hypoproteinemia, namely albumin and B-globulin, was reported to coincide with the increase of fat in the liver in Kwashiorkor (3).

This study was planned to investigate fat mobilization in Kwashiorkor as reflected on the plasma nonesterified fatty acids as well as the distribution of mono- and polyenoic acids in this lipid fraction (NEFA).

MATERIALS AND METHODS

Seventeen cases of classical Kwashiorkor infants of low social class attending the Cairo University Children Hospital were selected for the study. Their age was between 1-4 years. Thirteen normal children of the same age group were also studied. Fasting blood samples were collected from the internal jugular vein on the day following their admission to the hospital. The samples were kept in ice, analyzed for plasma NEFA by Dole's method (4) and fractionated for mono- and polyenoic acids by Abdel-Wahab's method (5) as follows: 0.3 ml aliquots of the heptane extracts containing the NEFA (equivalent to 0.1 ml plasma) were spotted on Whatman No. 1 filter paper strips previously impregnated with 5% (w/v) solution of silicone in ether according to the method of Schlenk et al. (6). The chromatograms were developed in acetic acid-H_2O system for 16 hr, dried and iodinated in a

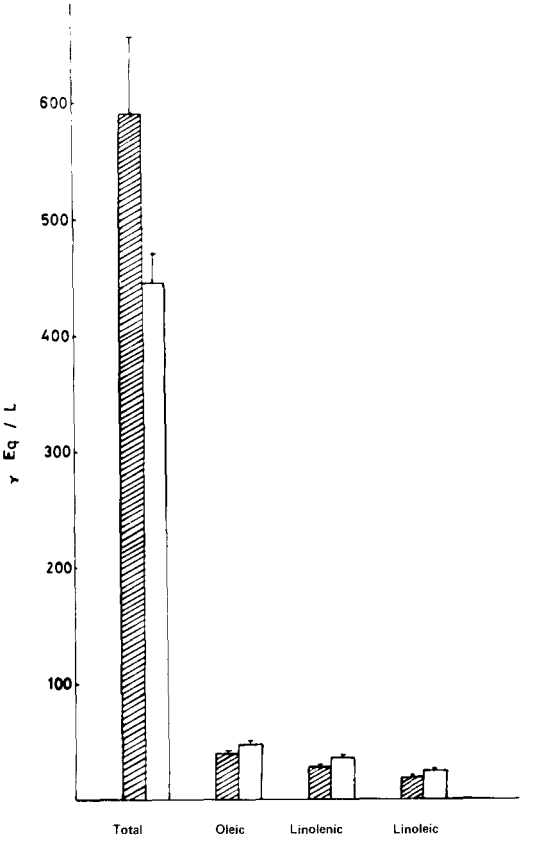

FIG. 1. Mono- and polyenoic fatty acid distribution in plasma nonesterified fatty acids in Kwashiorkor and normal infants. ▨ Normal; □ Kwashiorkor.

TABLE I

Mono- and Polyenoic Fatty Acid Distribution in Plasma Nonesterified Fatty Acids in Kwashiorkor and Normal

No. of cases	Total NEFA	Oleic		Linoleic		Linolenic	
	μEq/L	μEq/L	%	μEq/L	%	μEq/L	%
Normal (13)	591 ± 66	39.72 ± 3.38	7.33 ± 0.96	18.58 ± 0.50	3.45 ± 0.46	27.44 ± 2.64	5.23 ± 1.00
Kwashiorkor (17)	445 ± 32	48.74 ± 3.19	12.22 ± 1.36	24.64 ± 1.56	6.30 ± 0.70	35.63 ± 2.45	8.46 ± 0.69
P <	0.05	0.005		0.0025		0.01	

mixture of KIO_3 and KI containing 100 μCi of $K^{131}I$. The iodinated strips were dried in hot air to allow for evaporation of excess radioactive iodine and scanned in an automatic scanner (Frieske and Hoepfner GMB 452). Standard solutions of oleic, linoleic, linolenic and arachidonic acids (Sigma) were similarly treated. The data obtained were analyzed by the Student's t test.

RESULTS

The data obtained are given in Table I and Figure 1. Plasma NEFA levels were found to be much lower in Kwashiorkor infants (445 μEq/L ± 32) than in normals (591 μEq/L ± 66). The difference amounted to 146 μEq/L which is statistically significant ($P < 0.05$). Oleic, linoleic and linolenic acids were detected in measurable amounts in plasma NEFA fractions obtained from Kwashiorkor and normal infants, but were much higher in Kwashiorkor. The differences are highly significant. Arachidonic acid was hardly detected in either group and the radioactivity was always somewhat higher than the background.

DISCUSSION

The data presented in this study showed a statistically significant diminution in the total plasma NEFA concentration in Kwashiorkor than in normal infants. This is contradictory to the claim of Lewis et al. (7,8), who gave elevated NEFA levels in Kwashiorkor.

In Kwashiorkor the diet is very rich in carbohydrate, usually low in fat and very poor in protein. Such conditions favor fatty infiltration as they induce a fault in the transport of fat from the liver to the peripheral tissues due to reduced synthesis of lipoproteins or their precursors apoproteins. Such findings were confirmed by Monckeberg (3).

Various hormonal changes were reported in Kwashiorkor by several workers. Pathological and biochemical evidences for depression of suprarenal function were reported (9-12), including low blood eosinophil counts, diminished urinary excretion of 17-ketosteroids and 17-hydroxysteroids and glandular atrophy. Evidence of pituitary hypofunction in Kwashiorkor and protein malnutrition in general was also described (13,14). The thyroid gland is also affected in Kwashiorkor but not to the degree of producing clinical evidence of hypothyroidism (14). Low BMR, reduced ^{131}I uptake and low serum PBI were reported.

Thus, in Kwashiorkor it is possible that the depression of suprarenal, pituitary and thyroid functions, together with the possibility that there is inadequate plasma albumin for NEFA carriage may be the cause of the recorded decrease of plasma NEFA levels in our cases, than normal infants.

The distribution of mono- and polyenoic fatty acids in the plasma NEFA showed also a wide variation from normal, the differences from normal were always highly significant. The raised levels of the polyenoic fatty acids in Kwashiorkor can be attributed to the needs for these acids for the synthesis of fat, and/or be due to the preferential utilization of polyunsaturated fatty acids in the mobilization of other lipid components.

The higher levels of the polyenoic fatty acids encountered in our series are contradictory to the claim of other authors (15,16) who reported lower values or even values more or less similar to the normal. In our series NEFA fraction was examined while in other reports the serum total lipids were analyzed.

One can conclude from the previously discussed data that the excess carbohydrate in the diet of Kwashiorkor in addition to the state of hyperinsulinism, whether absolute or relative due to the deficiency of the pituitary, suprarenal and thyroid hormones, are responsible for the adequate lipogenesis and abundant of adi-

pose tissue fat with consequent decrease in NEFA levels in plasma. Still the endocrinal imbalance evident in Kwashiorkor may affect the distribution of saturated and unsaturated fatty acids by stimulation of differential release of depot fatty acids inhibiting the release of saturated acids.

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SHORT COMMUNICATIONS

Free Fatty Acid Production in Cerebral White and Grey Matter of the Squirrel Monkey

ABSTRACT

The free fatty acid level of squirrel monkey brain was shown to rise very rapidly after the blood supply to the brain was cut off, in agreement with previously reported data for rat brain.

In previous communications we described a rapid, linear and unique enlargement of the rat brain free fatty acid (FFA) pool as a consequence of a few minutes of ischemia and convulsions (1,2).

To ascertain whether this is a peculiar phenomenon linked to the rat brain or it is also present in other species, we have investigated the brain FFA pool size after varying periods of postdecapitation in the squirrel monkey; at the same time we have determined the FFA content of grey and white matter.

Squirrel monkeys (*Saimiri sciurens*) were used in this study. They were decapitated as soon as possible after capture from the housing cage. The samples were excised with a scalpel and homogenized in $CHCl_3$-CH_3OH 2:1 (v/v) at preselected time intervals (Table I). The first group of samples represents the fastest time interval possible. The procedures followed for the extraction of the lipids, and isolation and determination of FFA have previously been described in detail (2,3). Since the tissue samples were rapidly homogenized after removal and no time was spent in weighing, the results are expressed as micrograms of free fatty acids per 600 μg of phospholipid P. The latter was measured by the method of Bartlett (4).

The results shown in Table I indicate that during the first few minutes following shutting off the blood supply, a striking and linear production of FFA occurs in monkey grey matter. The data also show a much slower appearance of FFA in white matter. This suggests that the ischemia induced phenomenon is present in two widely different animals and that there is a highly significant difference in the capability of production of FFA in two parts of the nervous tissue analyzed. These results support our previous suggestion that myelin is not the main source of brain FFA (2).

Even when the white matter increment is slower than that occurring in grey matter, the FFA production of the former seems to be higher than the expected rate. Since the predominant FFA generated in rat brain were unsaturated and arachidonic acid was a main component, it was thought that some active polar lipids of structures other than myelin may well be the major source of the free acids (2). The increment of the FFA in ischemic monkey white matter may well be related to other structures than myelin such as axonal and mito-

TABLE I

Effect of Postdecapitation Ischemia on the Free Fatty Acid Content of Grey and White Cerebral Matter

	Samples[a]			
Tissue samples	92(88-94)	143(140-145)	205(200-208)	470(466-473)
Cerebral cortex[b]	108[c] ± 4.1[d](6)[e]	151 ± 4.3(5)	198 ± 5.3(4)	248 ± 5.8(6)
White matter[f]	---	---	53 ± 6.1(5)	114 ± 7.6(6)

[a]Each number represents the time intervals in seconds between decapitation and homogenization in $CHCl_3$-CH_3OH 2:1 (v/v). The numbers between parenthesis are the range.

[b]Cerebral cortex obtained from the parietal lobe.

[c]The results are expressed in micrograms of free fatty acids per 600 μg of phosphorus.

[d]Values are means ± standard error of mean.

[e]Figures between parenthesis indicates the number of samples.

[f]White matter excised from beneath the above mentioned cortical sample.

chondrial membranes. It was reported that autolysing human white matter contains increased levels of FFA several hours after death while fresh white matter have only traces (5). On the other hand, foci of encephalomalacic areas of human brain showed an augmentation of FFA (5,6) as in the case of malignant tumors of human brain (7). Rouser et al. (8) have indicated that the FFA content of a tissue lipid extract may be used as an indication of the extent of degradation and autolysis. In another study, Rouser et al. (9) present figures of the FFA content of human brain samples that represent values much higher than those obtained for animal brains (1-3). From these reports and the present study it is concluded that a very rapid production of FFA takes place in ischemic mammalian brain and that such phenomena take place mainly in grey matter.

A clear distinction should be made between the very early phase of ischemia, when such rapid production occurs, and the postmortem autolytic degradation of lipids during the irreversible phase of oxygen deprivation.

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[Received October 12, 1970]

The Fatty Acid Composition of Depot Fat in Childhood: II. A Comparison of Superficial and Deep Fat

ABSTRACT

The fatty acid composition of the triglycerides in samples of depot fat obtained from superficial abdominal wall and from deep properitoneal sites in children has been determined. Although no clear pattern was observed, differences in individual subjects could usually be demonstrated between composition at the two sites. Although the differences in most instances were relatively small, these findings provide further evidence against the concept of the uniform composition of human depot fat.

In 1901, Henriques and Hansen (1) showed that in the hog, fat from superficial depots was more unsaturated, and therefore less hard, than fat from deep body cavities such as perirenal,

TABLE I

Individual Differences in Percentage Fatty Acid Composition Between Superficial and Deep Fat in 17 Subjects[a]

	Fatty acid							
	12:0	14:0	16:0	16:1	18:0	18:1	18:2	18:3
Super. > Deep	1	2	4	6	4	7	4	3
No difference	10	8	7	8	8	7	8	11
Deep > Super.	6	7	6	3	5	3	5	3

[a]Significance is $p < 0.01$.

chondrial membranes. It was reported that autolysing human white matter contains increased levels of FFA several hours after death while fresh white matter have only traces (5). On the other hand, foci of encephalomalacic areas of human brain showed an augmentation of FFA (5,6) as in the case of malignant tumors of human brain (7). Rouser et al. (8) have indicated that the FFA content of a tissue lipid extract may be used as an indication of the extent of degradation and autolysis. In another study, Rouser et al. (9) present figures of the FFA content of human brain samples that represent values much higher than those obtained for animal brains (1-3). From these reports and the present study it is concluded that a very rapid production of FFA takes place in ischemic mammalian brain and that such phenomena take place mainly in grey matter.

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No difference	10	8	7	8	8	7	8	11
Deep > Super.	6	7	6	3	5	3	5	3

[a]Significance is $p < 0.01$.

TABLE II

Detailed Results in Three Representative Subjects[a]

	Fatty acid							
	12:0	14:0	16:0	16:1	18:0	18:1	18:2	18:3
2 1/2 months old male								
Super.	4.17(0.01)	7.12(0.03)	25.53(0.15)	6.05(0.01)	4.07(0.06)	33.17(0.13)	16.54(0.02)	1.54(0.07)
Deep	5.60(0.04)	6.89(0.06)	20.40(0.22)	3.38(0.01)	4.40(0.02)	31.94(0.13)	24.79(0.03)	1.20(0.04)
p <	0.1	5	0.1	0.1	1	1	0.1	5
13 years old male								
Super.	0.61(0.00)	3.33(0.01)	19.46(0.11)	4.44(0.02)	9.04(0.03)	44.57(0.15)	9.41(0.09)	3.99(0.11)
Deep	0.86(0.01)	3.97(0.02)	19.19(0.02)	5.06(0.04)	8.61(0.03)	42.16(0.18)	9.97(0.04)	4.22(0.10)
p <	0.1	0.1	ns	0.1	0.1	0.1	1	ns
17 years old female								
Super.	0.11(0.00)	1.57(0.03)	15.74(0.01)	11.23(0.02)	0.83(0.07)	53.16(0.05)	11.42(0.02)	3.39(0.06)
Deep	0.26(0.01)	1.98(0.03)	19.75(0.04)	10.90(0.03)	1.26(0.03)	48.26(0.03)	11.95(0.02)	2.98(0.02)
p <	0.1	0.1	0.1	1	1	0.1	0.1	1

[a]Percentage composition, mean and standard error on triplicate estimations. Probability, p x 100.

pericardial or omental tissues. They concluded that the temperature of the tissue was the determining factor, since unsaturated fats have a lower melting point. Further studies by Dean and Hilditch (2) confirmed this, although it was found that the increase in unsaturation was confined to the most superficial subcutaneous layers. The subject is reviewed by Hilditch and Williams (3). Accordingly it has been customary to consider that such variations in composition occur in all mammals, perhaps excluding man.

We have recently demonstrated that there is a continuing change in the fatty acid composition of human subcutaneous abdominal wall fat from birth towards puberty (4). Since we have shown age-dependence in superficial fat composition, it is necessary to avoid this variable in a comparative study by utilizing paired samples from individual subjects.

Small (0.1 to 0.5 g) samples of subcutaneous abdominal wall and deep properitoneal (subserous) fat were obtained at the time of surgery, usually for elective hernia repair. Consequently there were 13 male but only 4 female subjects. Ages ranged from early infancy to 17 years. The tissue was cleaned of gross blood and stored in 0.9% saline containing 0.5% hydroquinone as an antioxidant; it was frozen as soon as possible, always within 1 hr. Two or usually three portions weighing from 30 to 60 mg were prepared for fatty acid analysis by gas liquid chromatography as described previously (4).

While each sample may not necessarily be as representative of the tissue as separately collected samples might, this method provides some estimate of variation due to analytical technique. As will be noted, such variation was extremely small.

A summary of our results is presented in Table I. Since we have shown that at least superficial fat composition is age-dependent in childhood, it is not possible to study group findings (4). Accordingly, the mean and standard error of the mean for individual superficial and deep samples were calculated, making use of the duplicate or triplicate analyses. The means were then compared using the "t" test.

Between superficial and deep composition, every subject showed a significant difference in percentage composition of at least one fatty acid. In 32% of instances the difference was highly significant ($p<0.001$), and in 68% it was probably significant ($p<0.05$). However the differences appear to be random, in that they are not related to the age or sex of the subject, and in a given fatty acid the level in superficial fat may be greater or less than in deep fat. Differences were most common in linoleic acid (18:2) and least common in linolenic (18:3).

The detailed findings in three representative subjects are shown in Table II. The level of stearic acid in the third subject is lower than any in our previous series (4) although the range in post-adolescent females (2-8%) was wide: this is presumably of dietary origin although no history of unusual dietary habits was evident.

The literature with regard to the effect of sample site in human adipose fatty acid composition is relatively scarce, in part due to the tendency to sample from only one site in surveys. The use of the Hirsch aspiration technique has focused attention, in the living subject, on buttock fat. Most papers do not provide data on children. In those studies where multiple sites were sampled, most authors found no difference between the fatty acid proportions in superficial (trunkal) and deep (mesenteric, omental or properitoneal) adipose tissue (5-8).

Kingsbury et al. (9) reported a slightly higher level of stearic acid (18:0) in perinephric fat as compared with tissues from the abdominal wall or buttock regions in adults, but considered the difference almost insignificant.

Hegstedt et al. (10), in a study of fatty acid patterns in various body sites in subjects from various parts of the world, showed that there was a little variation with site in some adult samples, but no particular pattern emerged.

McLaren et al. (11) showed that in general fat from deep intra-abdominal sites contained more stearic (18:0) and less palmitoleic (16:1) acid than samples from superficial, mainly limb sites. However there is definite evidence suggesting that fat from the distal limb (5,6,11,12) or intra-articular (11) sites is of quite different fatty acid composition than from the rest of the body.

Baker (13) has shown a higher level of linoleic (18:2) acid in perirenal fat compared to subcutaneous fat in premature infants, but not in normal newborns or older children.

In general the data in adults has suggested no real compositional differences in triglycerides from various trunkal sites. The present results definitely show such differences in individual subjects; however, the data give no lead as to the mechanisms of such differences, as they do not seem to be related to age or sex, and vary from one individual to another.

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Sterols in Chloroplasts: A Description of Two Qualitatively Distinct Forms[1]

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ABSTRACT

Chloroplasts from eight plant species have been extracted sequentially with petroleum ether (PE) and acetone (A) and the extracts analyzed for sterols. Cholesterol was a component of all fractions analyzed but it was found that the A fraction of each species contained relatively much more cholesterol than the PE fraction. Other components of the PE sterol fractions were plant sterols typical of the species examined and were quantitatively markedly different from those in the corresponding A fractions. The yield of sterol from chloroplasts was found to be 0.02-0.03% and the ratio of the PE/A fractions to be 5:1-19:1.

INTRODUCTION

Several earlier references, detailed by Mercer and Treharne (1), indicated that the occurrence of sterols in chloroplasts had been a subject for dispute. However they were able to demonstrate clearly the presence of sterols in highly purified chloroplast preparations from *Phaseolus vulgaris* and to show that, while sterols were apparently leached out by hexane during the isolation process, further sterols could still be extracted, using acetone, after five washings with hexane. The acetone extracted sterols apparently consisted predominantly of cholesterol, while the hexane extracted fraction consisted largely of C-28 and C-29 sterols (1,2). It has also been found, using gas liquid chromatography (GLC), that chloroplasts from *Spinacia oleracea* contain cholesterol together with α-spinasterol and Δ^7-stigmastenol (3) and that chloroplasts from *Zea mays* may be divided into two fractions (4). In the first of these, esterified sterols, cholesterol constituted 50% of the mixture and β-sitosterol 35%, while in the second fraction, unesterified sterols, the composition was: cholesterol 2-3%, campesterol ca. 13%, stigmasterol ca. 25% and β-sitosterol ca. 59%. No attempt was made with these two species to extract the chloroplasts sequentially with solvents of differing polarity.

It has been reported that sterols are synthesized outside the chloroplast and subsequently transferred to the developing organelle during greening of etiolated seedlings (5) and that this

[1]One of 12 papers to be published from the "Sterol Symposium" presented at the AOCS Meeting, New Orleans, April 1970.

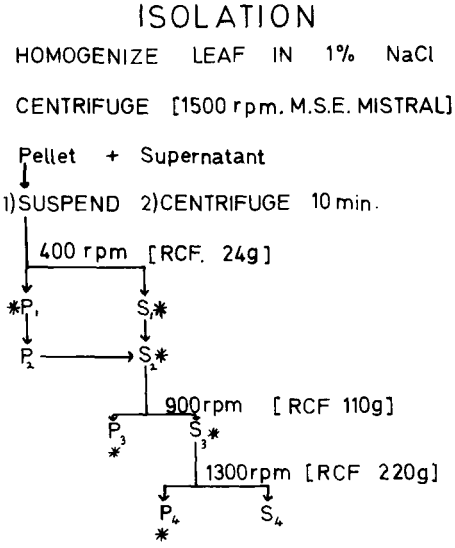

FIG. 1. Pellets P_3 and P_4 contained chloroplast and were recentrifuged three times to remove contaminating particles. All other fractions were discarded.

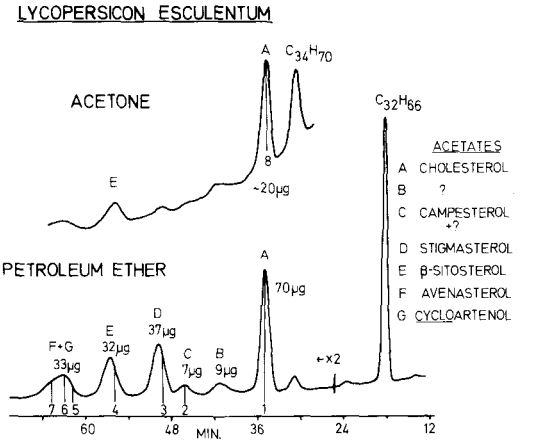

FIG. 2. Acetylated sterols from chloroplasts. $C_{32}H_{66}$ was added as internal standard and $C_{34}H_{70}$ was an impurity in this compound. Quantities of each sterol, in µg, are indicated. Mass spectral scans were taken at the points indicated by Arabic numerals below the peaks.

TABLE I

Sterols in Chloroplasts

Species	Extract[a]	Sterols[b], %							
		1	2	3	4	5	6	7	8
S. alba	PE	4.0	Trace	11.2	---	72.5	---	---	11.5
	A	23.0	25.0	Trace	Trace	34.0	---	10.6	7.4
L. sativa	PE	12.0	---	6.4	27.8	49.2	---	---	4.2[c]
	A	43.6	--	17.0	8.7	26.2	---	---	4.5[c]
P. vulgaris	PE[d]	2.0	--	9.0	30.0	59.0	---	---	---
	A	11.0	---	5.2	25.0	58.8	---	---	---
B. vulgaris	PE	7.2	---	Trace	7.3	52.6[e]	52.6[e]	32.9	Trace
	A	53.6	---	Trace	8.5	22.6[e]	22.6[e]	15.3	Trace
S. oleracea	PE	30.0	---	---	---	60.5[e]	60.5[e]	---	9.5[c]
	A	50.0	---	10.0	---	40.0[e]	40.0[e]	Trace	Trace
S. tuberosum	PE	30.3	14.6	Trace	13.1	42.0	---	---	---
	A	~40.0	?	---	?	~40.0	---	---	---
L. esculentum	PE	36.5	4.8	3.9	19.1	17.2	---	17.4	17.4[f]
	A	80.0	---	---	Trace	20.0	---	---	---
N. tabacum	PE	11.4	5.9	16.5	29.0	30.5	---	---	6.7
	A	61.7	---	11.8	6.8	19.7	---	---	---

[a]PE, petroleum ether extract; A, acetone extract.

[b]Sterols: 1, cholesterol; 2, brassicasterol; 3, campesterol; 4, stigmasterol; 5, β-sitosterol; 6, α-spinasterol; 7, Δ^5-avenasterol; 8, Δ^7-stigmastenol; and cycloartenol.

[c]Δ7-Stigmastenol.

[d]Chloroplasts from this species were only extracted 6 x PE.

[e]β-Sitosterol and α-spinasterol were only partially resolved by GLC. The latter predominated in PE extracts and the former in A extracts.

[f]Cycloartenol.

process is controlled by the phytochrome system (2). During studies in the Cruciferae it was found that, in those cases where cholesterol was absent from the seed, this sterol was biosynthesized very rapidly following germination (6). Cholesterol, which is known to occur in several plant species (7,8), may play a role in the development and function of chloroplasts. This paper describes an investigation into the sterol composition of chloroplasts isolated from a number of sources.

EXPERIMENTAL PROCEDURES

The method is isolating chloroplasts is outlined in Figure 1. Chloroplasts were found in both the P3 (110 g) and P4 (220 g) fractions and were further cleaned by suspending and recentrifuging the pellets in 1% sodium chloride solution three more times. The chloroplast pellets were examined microscopically for contaminants; few bacteria could be detected (<1%) and the principal contaminant was always found to be starch grains. The grana were clearly visible in all cases and the chloroplasts appeared to have largely retained their shape and contents. Chloroplasts were obtained in the indicated fractions from the following species: *Sinapis alba*, 110 g, (Hurst, Gunson, Cooper, Taber; Witham Essex); *Lactuca sativa*, 110 g, cv Grand Rapids (Page Seed Co., Greene, New York, U.S.A.); *Phaseolus vulgaris*, 110 g, cv Kentucky Wonder (Hurst, Gunson, Cooper, Taber; Witham, Essex); *Beta vulgaris*, 110 and 220 g, cv Spinach Beet (Hurst, Gunson, Cooper, Taber; Witham Essex); *Spinacia oleracea*, 110 and 220 g (McPhee, Moss Street, Paisley, Renfrewshire); *Solanum tuberosum*, 110 g, mixture of five varieties; *Lycopersicon esculentum*, 110 and 220 g, cv Money Cross (Pine Tree Nurseries, Parr, Cornwall); and *Nicontiana tabacum*, 110 g, cv White Burley (Dobbie and Co. Ltd., P.O. Box No. 113, Edinburgh).

Plants were cultivated as follows: *S. alba* in pots in a growth environment chamber (6) under alternate periods of 8 hr continuous illumination and 16 hr darkness to prevent flower induction; *B. vulgaris* was grown outside in the departmental experimental garden; all other species were grown in pots in a greenhouse using supplementary lighting during the winter (*L. sativa* and *S. tuberosum*). Watering was carried out using a modified Hoagland's nutrient solution (6). Plant material of various

ages was stored frozen or used freshly harvested.

Sterols were isolated as follows: (a) dry chloroplasts: vacuum, Na_2SO_4; (b) extraction: 15 x petroleum ether and 6 x acetone; (c) saponify (NaOH); (d) digitonin precipitation of nonsaponifiable fraction; (e) add $nC_{32}H_{66}$, BSA; (f) GLC. It was felt that the best drying technique for the chloroplasts was to vacuum desiccate until most of the occluded water had been removed from the pellet and then to add petroleum ether (PE) and anhydrous sodium sulfate with stirring (spatula) until a fine powder was formed. Vacuum desiccation alone produced a hard pellet which was not easily broken up for extraction. Extraction with PE (b p 40-60 C) was achieved by heating for 5 min at 40-50 C followed by centrifugation (1000 *g*) and decantation. The process was repeated 15 times to give the PE fraction and was followed by six extractions with acetone (A) to give the A fraction. It was necessary to extract these numbers of times because material was still being extracted after 10 extractions with PE (as indicated by the results for *P. vulgaris* in Table I where only six extractions were used to produce the PE fraction).

Sterols were isolated by previously described techniques (6,9) and were analyzed by GLC using 3% OV-17 coated on Gas Chrom Q (Applied Science Laboratories pretested packing) as stationary phase at 255 C. Prior to GLC a known amount of $nC_{32}H_{66}$ was added as an internal standard for the analysis, followed by bistrimethylsilyl acetamide (BSA) to prepare trimethylsilyl ethers. Samples were ready for analysis after 10 min. Using standard mixtures, cholesteryl trimethylsilyl ether and $nC_{32}H_{66}$ produced similar response-weight ratios in the flame ionization detector (Pye 104 model 14) and the ratio of responses remained constant over a wide range of sample sizes. For the purpose of quantitative analyses, it was assumed that all sterols were eluted from the column with equal efficiency and that they all exhibited similar responses in the detector (10).

GLC-mass spectrometry was carried out using an LKB 9000 mass spectrometer fitted with a 1% OV-17 column at 250 C operated under previously described conditions (11). For this analysis, sterols from chloroplasts of *L. esculentum* were used and were converted to acetates prior to analysis (Fig. 2).

RESULTS

Table I contains a list of the percentages of each sterol detected by GLC in both the PE and A fractions from chloroplasts isolated from eight species, representatives of five families, studied in this work, and a representative result obtained as the acetates, from *L. esculentum* is illustrated in Figure 2. The assignments in Table I are based upon known GLC properties and known sterols in species examined. They were confirmed by combined GLC mass spectrometry for *L. esculentum*. It is apparent that cholesterol is present in all fractions in both series and represents a consistently higher proportion of the total sterol in the A fractions when compared with the corresponding PE fractions. The range of proportion of cholesterol in the PE fractions (4-36%) approximately parallels that observed by GLC for cholesterol in various green tissues which have been subject to PE extraction and analysis by GLC (9). Although lower than described in the literature for *Ph. vulgaris*, the general range of cholesterol proportions in the A fractions is comparable to that noted by Mercer and Treharne (1). It should be stressed that in the present work, chloroplasts from *Ph. vulgaris* were not completely extracted with PE prior to acetone extraction and therefore this result must be set aside in any detailed discussion. *S. alba* is remarkable in having the least cholesterol in the A fraction and for having a significant quantity of a sterol presumed to be brassicasterol (a sterol typical of the tribe bassicinae in the family Cruciferae and known (6) to be present in seed of *S. alba*). It is noteworthy that brassicasterol is not detectable in oven-dried leaf material of some members of the Cruciferae after the seedling stage of growth until the plants have flowered and start to fruit. Thus it is possible that this component of chloroplasts from *S. alba* may originate in the sterols stored in the seed and be incorporated into the A fraction of the developing chloroplast along with Δ^5-avenasterol, which is also present in the seed of this species. This hypothesis might be verified using another species of the Cruciferae with a differing seed sterol fraction (e.g. *Lepidium sativum, Cheiranthus cheiri* (6). Also, brassicasterol is the only sterol from

TABLE II
Quantitative Analyses

Species	Sterol yield PE (μg)	Sterol yield A (μg)	Ratio, PE/A	Yield[a], %
N. tabacum	76	4	19	---
S. tuberosum	39	2	19	---
L. esculentum	188	30	6.3	~0.02
S. oleracea	53	10	6.3	~0.03

[a]Dry weight of chloroplasts.

higher plants known to have the (24*S*) configuration but that other (24*S*) sterols are often found in green algae. While this also represents a possible area for further study, at the present time, GLC is not a suitable method for distinguishing the stereochemistry at C-24 in most sterols; the structural assignments in this work have been made with this limitation in mind.

Campesterol, or its C-24 isomer, 22-dihydrobrassicasterol, was only a minor component of all fractions examined but C_{29} sterols were commonly present in all fractions. The principal C_{29} sterol was β-sitosterol except for the PE fractions of the two members of the Chenopodiaceae, *B. vulgaris* and *S. oleracea*, where the main sterol was α-spinasterol. While stigmasterol was often detected, Δ^5-avenasterol [(24Z)-stigmasta-5,24(28)-dien-3β-ol] (12) was only detected in measurable quantity in the A fraction from *S. alba* and both fractions from *B. vulgaris*. It was also detected in a partially resolved, mixed peak from *L. esculentum* when the second component was shown, by GLC mass spectrometry, to be cycloartenol. GLC mass spectrometry confirmed the identity of most of the compounds present in both fractions of this species; scans were obtained at the points indicated in Figure 2.

Table III lists the results of quantitative analyses which give an indication of the relative proportions of the PE and A fractions. The ratios PE/A are comparable to that obtained by Mercer and Treharne for *Ph. vulgaris*, but the overall yield of sterol appears to be appreciably lower than theirs (1).

The PE/A ratios and the general composition of the two fractions obtained in the present results parallel closely the results quoted by Kemp and Mercer for *Z. mays* (4). Their unesterified sterol fraction corresponded approximately in relative amount and composition to the PE fractions and their esterified sterol fraction corresponded closely to the A fractions. This correspondence prompted the idea that the PE fraction of chloroplast sterols might be unesterified and readily accessible to extraction by lipophilic solvents and that the A fraction might be a deeply embedded ester fraction surrounded by a hydrophilic layer and therefore comparatively resistant to extraction by lipophilic solvents. To test this theory, chloroplasts from *P. vulgaris* were extracted and subjected to separate analysis for free and esterified sterol. Contamination with fungal spores made the isolation difficult and reduced the yield so that analysis was not complete. However, the results suggested that the above theory did not hold, as sterols were detected in both the esterified and unesterified parts of the PE fraction. The esterified sterol seemed to be largely cholesterol and further work along these lines may provide additional information.

It has recently been reported (13) that sterols in the organism *Euglena gracilis* exist in four forms. In both the green (light grown) and white (dark grown) forms of this organism sterols occurred in unesterified, esterified, water soluble-acid hydrolyzable and water soluble-alkaline pyrogallol labile (14) forms; the two latter forms are not extractable with acetone or chloroform-methanol solvents. In the white form, all four fractions contained about 15% of cholesterol, but in the green form there was a high specific binding of cholesterol constituting 80% of the sterols in the alkaline pyrogallol form (37% of the total) and only 1% of the unesterified sterols (57% of the total). It is clear that light has a marked effect upon the binding of sterols in *E. gracilis*. It appears also that the selectivity of this effect is at least as marked as the selective location of cholesterol in the esterified sterol fraction of chloroplasts from *Z. mays* (4) and in the A fractions of the present investigation. While neither the location of sterols in organelles from *Euglena* nor the occurrence of water soluble forms of sterol in chloroplasts have been described, further study may provide much information on the binding of sterols in general and on the occurrence of sterols in chloroplasts in particular.

ACKNOWLEDGMENTS

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Regulation of the Sequencing in Sterol Biosynthesis[1]

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ABSTRACT

The evolutionary development and sequencing of events in the biosynthetic pathway leading to sterols is reviewed and illustrated by recent experiments from the author's laboratory.

It has now become apparent that the de novo biosynthesis of sterols occurs by essentially the same pathway regardless of the organism in which it takes place. Some of the reasons are: (a) mevalonic acid serves as the precursor of sterols in mammals (1), fungi (2,3), and lower and higher photosynthetic plants, e.g., phytoflagellates (4), brown algae (5), ferns (6), gymnosperms (7), and angiosperms (8,9); (b) squalene is formed in and metabolized to sterols in both the animal (10,12) and nonphotosynthetic (2,13) and photosynthetic (14,15) plant kingdoms; (c) the labeling pattern in squalene is the same in both animals (16,17) and plants (18), when 2-^{14}C-mevalonate is used as substrate, indicating similarities both in the gross character of the pathway and in the stereochemistry of the isomerization of Δ^3- to Δ^2- isopentenyl pyrophosphate (18); (d) in the course of the latter isomerization as well as in the polymerization of the C_5-units, which leads to farnesyl pyrophosphate and then to squalene, an H-atom is eliminated from the same side of the molecule in both nonphotosynthetic (19) and photosynthetic (20) plants and in animals (21), since in both cases (4R)-4-$^3H_1$-mevalonate leads to retention of the label. Therefore, the biosynthetic problem has resolved itself into questions relating to the development and regulation of the sequence of events in this general pathway.

About a decade ago, at the time when most of the available evidence was taken from the most highly evolved of the animals, i.e., mammals, we began an examination of the sterol pathway in fish to determine whether there were any obvious differences at this much lower level of vertebrate development. We were able to show (22) that mevalonate, squalene and lanosterol were intermediates and that, as had been found with rats, mevalonate was utilized much faster than its precursor, hydroxymethylglutarate. More recently we (23), as well as Reitz and Hamilton (24), have demonstrated that sterols are present in blue-green algae which have been grown on a salt medium lacking in steroidal components. The sterols isolated must clearly have been biosynthesized in the algae. Blue-green algae are believed (25,26) to have arisen as much as two billion years ago, and show, by being procaryotic (27), a very primitive type of intracellular organization. In addition the isopentenoid trimer, farnesane, which probably is derived from farnesol, has been detected, along with other isopentenoids, (26,28,29) in the Soudan shale, dated at 2.5 billion years ago. It therefore seems very likely that the steroid pathway evolved more or less coincidentally with the evolution of life itself. In examining steroid biosynthesis in organisms presently extant, whatever the differences might be, we are likely to find that they are differences only in detail.

Several major kinds of detail can vary. In the first place, there can be alternative portions of the same general pathway in a manner somewhat analogous to the shunt so well described in glycolysis. Secondly, there can be additions to or deletions from the pathway producing, respectively, new metabolites or a dietary requirement for precursors or intermediates. The latter is especially well documented in insects (30,31 and references cited therein). Thirdly, there can be differential control at a quantitative level, i.e., the number of molecules which pass through a given point in the pathway per unit time may be different from tissue to tissue, from organism to organism, and from time to time, and the manner in which this regulation is imposed may vary.

The first and most obvious influence on control at either the qualitative (structural) or quantitative (kinetic) level is the intrinsic chemistry of the substrate. In the steroid field this was perhaps first vividly demonstrated by the finding in Bloch's laboratory (32) that during conversion of lanosterol to cholesterol position 3 is oxidized to a carbonyl group and then reduced back to a carbinol group. The reason for this is clearly a mechanistic one in which loss of a C-atom from C-4 requires an electron sink in the β-position (C-3). Since good evidence for the intermediacy of the carboxyl

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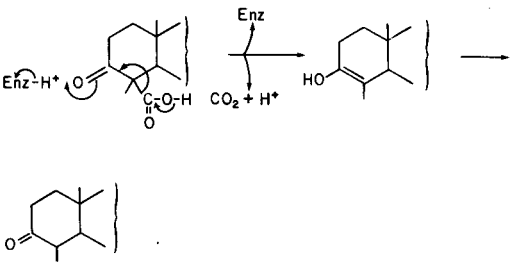
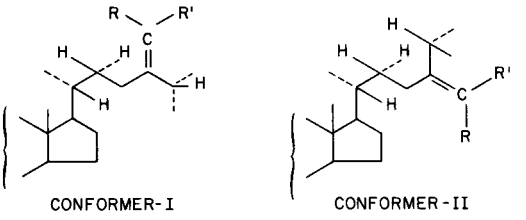
[1]One of 12 papers being published from the "Sterol Symposium" presented at the AOCS Meeting, New Orleans, April 1970.



Scheme 1. Role of 3-ketone in elimination.

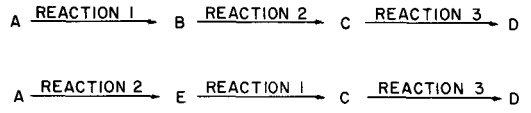


Scheme 2. Conformations under consideration for dehydrogenation at C-22,23.

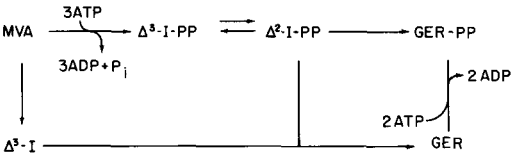
stage of oxidation has been presented by Gaylor and his associates (33), one can write the elimination as shown in the accompanying scheme (stereochemistry being ignored), which focuses attention on the role of the 3-ketone. The demethylated ketone is then reduced back to the equatorially oriented hydroxy compound which again, since the equatorial isomer is the more stable, probably has its origins (at least in an evolutionary sense) in the substrate's preferential chemistry. In collaboration with the group of investigators at Bryn Mawr College, we (34) have been able to present another example of how steroid metabolism can be controlled in this way. It is concerned with the stereochemistry about the $\Delta^{24(28)}$-bond and with its influence on dehydrogenation at C-22,23.

The Δ^{22}-bond is quite commonly present in plant sterols (stigmasterol, ergosterol, etc.). Conner, et al. (35) have now found that, along with a Δ^{7}-bond, it is also introduced into cholesterol by the protozoan *Tetrahymena pyriformis*. Since our own laboratory had available various derivatives of cholesterol in which C-24 was substituted as a result of studies on the mechanism of alkylation at this position (7,36 and references cited therein), we undertook an examination of the influence the substituent might have on the 22,23-dehydrogenation. We found that 24-methylenecholesterol readily undergoes dehydrogenation in the protozoan both at C-7,8 and C-22,23 to yield *trans*-$\Delta^{5,7,22,24,(28)}$-ergostatetraene-3β-ol. Its structure was demonstrated by mass spectroscopy, by the presence of appropriate absorption for the two conjugated diene systems in the UV region of the spectrum, by the presence of absorption at 967 cm^{-1} corresponding to a *trans*-Δ^{22}-bond, and by agreement of its melting point with that of the same compound previously isolated (37) from yeast. Conner et al. (35) had already shown that with cholesterol itself, the Δ^{22}-bond introduced has the *trans*-configuration. On the assumption that the dehydrogenation proper, i.e., removal of the H-atoms, takes place faster than do conformational changes, we can infer from the configurational data that the side chain must assume the s-*trans*-conformation about the C-22,23 bond. From other data available on the stereochemistry of elimination (38), it becomes apparent that the two H-atoms leaving C-22 and C-23 must lie on the same side of the plane passing through C-20, C-22, C-23 and C-24. If this is true, substituents on C-24 (C-25 and C-28) must also lie in this plane if minimal interference is to occur in formation of the enzyme-substrate complex. Conversely, any effect which forces these groups out of the plane should destabilize the complex and reduce the rate of hydrogenation. Scheme 2 shows that 24-methylene-cholesterol (R = R′ = H) can readily assume this favorable condition in Conformer-I. However, its isomer, Conformer II, which is derived by a rotation of 180° around the C-23,24 bond and by an adjustment of the rotational angle between C-24 and C-25, so that the H-atom in C-25 lies between those on C-22, ought to be less favorable because the *gem*-dimethyl groups project on either side of the central plane. Adjusting the C-24,25 angle so that one of the methyl groups lies between the H-atoms on C-22 would destabilize the system because of the strong interaction of the methyl H-atoms with those on C-22 which would force C-25 and C-28 out of the plane and, in turn, would destabilize the substrate-enzyme complex. The favorable conformation (Conformer-I) can occur even if R′ is CH_3 (e.g., in 28-isofucosterol), but not if R is CH_3 (e.g., in fucosterol).

Therefore 28-isofucosterol, but not fucosterol, would probably undergo the dehydrogenation at C-22,23, but both should undergo dehydrogenation at C-7,8, since this conformational problem should be irrelevant with respect to ring B. We submitted both of these substrates to incubation with *T. pyriformis*. As anticipated, while 28-isofucosterol led to *trans, trans*-$\Delta^{5,7,22,24,(28)}$-stigmastatetraen-3β-ol, fucosterol led to *cis*-$\Delta^{5,7,24,(28)}$-stigmastatrien-3β-ol. The structures of the two metabo-



Scheme 3. Alternative sequences by rearrangement of ordering of reactions.



Scheme 4. Possible physiologic significance of utilization of free geraniol (MVA = mevalonate, I = isopentenol, PP = pyrophosphate, and Ger = geraniol).

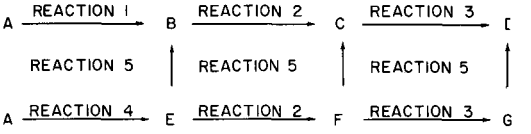
lites, previously unknown, were demonstrated by mass and light spectroscopy in an unequivocal fashion. Both showed typical $\Delta^{5,7}$-absorption at 272, 282 and 294 nm, but only the metabolite from 28-isofucosterol showed the presence of the $\Delta^{22,24(28)}$-chromophore by absorption at 237 nm.

The influence of the configuration about C-28 on the dehydrogenation is but one more facet in the larger constellation of chemical effects on metabolism. However, it may have a more specific meaning in steroid biosynthesis. There are reasons for believing (7,39) that the *trans*- rather than the *cis*-isomer of the 24-ethylidenesterols are involved in the pathway leading to 24-ethylsterols. If introduction of the Δ^{22}-bond were a prerequisite to reduction of the $\Delta^{24(28)}$-bond, then the intermediacy of the *trans*-isomer (Conformer-I, R' = CH_3) would be understandable, since it, rather than the *cis*-isomer, can readily proceed to the Δ^{22}-derivative. The fact that dehydrogenation of β-sitosterol to stigmasterol can be demonstrated (40) in a small yield does not contradict this suggestion, but the report that fucosterol yields clionasterol in a species of *Chlorella* (41) does. A final analysis of the pathway and of the influence of the configuration at C-28 on it will therefore have to await further investigation.

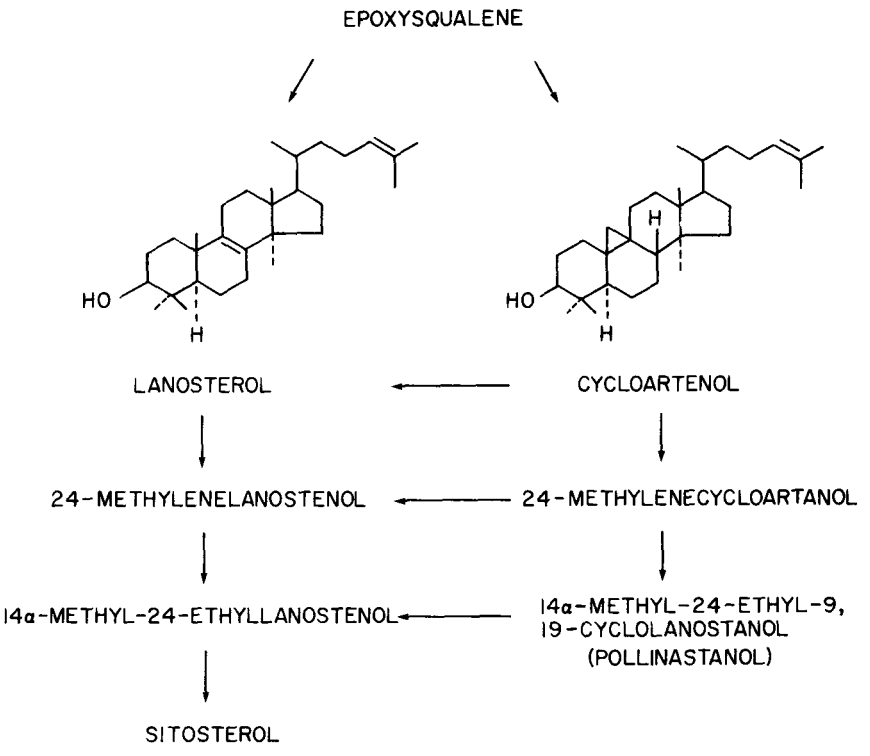
In addition to the intrinsic chemistry of the substrate, the chemistry and concentration of the enzyme is a factor in determining the outcome of a biochemical reaction and, consequently, the precise sequence in which the reactions occur. In the simplest case, alternative sequences can arise by placing a given set of reactions in a different order. This is illustrated in Scheme 3 in which Reaction 1 and Reaction 2 occur in that order in one sequence and in the reverse order in a second sequence. An example of this in the sterol field is the conversion of lanosterol to desmosterol and then to cholesterol or, alternatively, the conversion of lanosterol to 24,25-dihydrolanosterol and then to cholesterol. Reaction 1 is a set of transformations involving changes in the ring system, while reaction 2 is the reduction at C-24,25. This problem has been greatly expanded in the hormone field. We (42) have also been able to show that it exists in the early part of the pathway by demonstrating that free geraniol is phosphorylated and converted to squalene in a cell free system from germinating peas. Baisted (43) has also shown that free geraniol proceeds to squalene in vivo in this organism. A pathway involving free geraniol is different from the one believed to take place in, for instance, mammals because of the position of the kinase in the sequence. In mammals, mevalonate is phosphorylated (44) and the geraniol produced already bears the pyrophosphate group. If the utilization of free geraniol, which has also been observed in the carotenoid field (45), has any real significance, the pathway involving it is more complicated than that envisioned in Scheme 3, because Δ^2-isopentenol, its precursor and C-source for the gem-dimethyl end of the molecule, must be phosphorylated for energetic and mechanistic reasons. A possible pathway is shown in Scheme 4 in which the upper sequence is the normal one and the lower sequence the alternative one. It is also possible that the presence of a kinase for geraniol is nature's way of preventing loss of an intermediate by inadvertent hydrolysis, i.e., a sort of scavenging process.

A somewhat different type of alternative sequence may occur by the substitution of a new reaction for one in the original sequence instead of just a change in the order of the existing reactions. This is illustrated in Scheme 5 where Reaction 4 substitutes for Reaction 1. To proceed to the same end product in both sequences, still another reaction (Reaction 5 in Scheme 5) must be introduced. We may call this a "crossover" reaction, since it is a correction for the first substitution and directs the molecules from the second into the first sequence. A very important example of this has recently come to light.

In many cases, which appear to by photo-



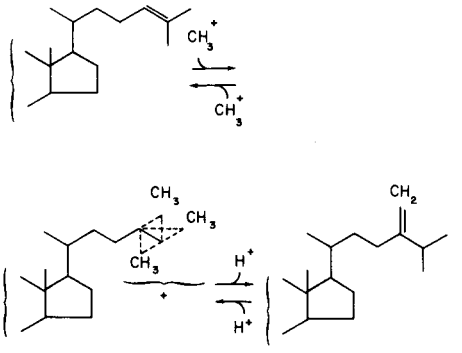
Scheme 5. Alternative sequences in which a new reaction is substituted.



Scheme 6. Alternative routes to phytosterols.

synthetic (46-50) rather than nonphotosynthetic (13,51), cycloartenol substitutes for lanosterol as the metabolite of epoxysqualene in the sterol pathway (Scheme 6). We (52) were able to show that in *Pinus pinea*, lanosterol proceeds to 28-isofucosterol which in turn proceeds (7) to 24-ethylcholesterol. However, we also showed (53) that both lanosterol and cycloartenol underwent alkylation in a cell free system from peas to yield the respective 24-methylene derivatives. Subsequently it was shown in other laboratories that tobacco leaves (54) and the photosynthetic microorganism, *Ochromonas malhamensis* (55), will convert either lanosterol or cycloartenol to 24-alkylsterols. Since mammalian tissue, and fungi (51), uses the lanosterol route, and photosynthetic plants appear to use the cycloartenol route, the question of how the routes are controlled arises. We therefore prepared 2-tritio-lanosterol and 2-tritio-cycloartenol and submitted both to incubation with a rat liver homogenate (G.F. Gibbons and W.R. Nes, unpublished data). Labelled cholesterol, which lost label upon conversion to the 3-ketone and submission to keto-enol equilibration, was formed in a 10% yield, but the labeled cycloartenol yielded neither labeled pollinastanol nor labeled cholesterol. This demonstrates that the enzymes in the sequence following lanosterol are quite specific and that no crossover enzyme (an isomerase) exists. Consequently, not only does primary control of the pathway reside in the nature of the epoxysqualene cyclase (Reaction 1, Scheme 5 as opposed to Reaction 4), but secondary control resides in the specificity of the enzymes later in the pathway as well as in the absence of the isomerase (Reaction 5, Scheme 5). On the other hand, in collaboration with a group of investigators at the University of Liverpool (Gibbons et al., unpublished data), we were able to show by stem feeding that in corn seedlings, which were shown to biosynthesize only cycloartenol, both lanosterol and cycloartenol (labeled in both cases either with tritium at C-2 to with ^{14}C by biosynthesis) served as substrates for the biosynthesis of the plants' dominant sterols (24-methyl- and 24-ethylcholesterol). In this case the control of the pathway must reside exclusively at the cyclization of epoxysqualene.

The problem of two competing reactions leading to a sequence in which no crossover reaction exists is also found in the reduction (56,57) vs. alkylation (7,36 and references cited therein) of the Δ^{24}-bond. While both reactions



Scheme 7. Equilibrium reaction for alkylation of the Δ^{24}-bond.

occur in plants, since both cholesterol and 24-alkylcholesterol are present (60), in mammals the presence of only cholesterol indicates that only reduction occurs. How is this lack of alkylation controlled? In principle, it could be due to several factors of which two are foremost: the lack of an alkylase or control of the availability of an appropriate cofactor which in this case would be S-adenosylmethionine (59) or both. Since the first step (53) in alkylation is the formation of a 24-methylenesterol, we have injected synthetic 28-^{14}C-24-methylenecholesterol intraperitoneally into rats (60; also, Thampi et al., unpublished data). If an enzyme for alkylation is absent, no reversal of alkylation can occur. On the other hand, if the alkylase is present and the equilibrium position is maintained on the side of dealkylation by cofactor control at the enzymatic site (by maintenance of the S-adenosylmethionine-S-adenosylhomocysteine equilibrium on the side of the latter), dealkylation should occur. In fact, 28-^{14}C-24-methylenecholesterol led in a 19% yield to labeled cholesterol. This was demonstrated by a variety of techniques including gas liquid (GLC) and argentation chromatography both of which readily separate the substrate and product. The yield was much too high to be accounted for by removal of C-28 and resynthesis. Furthermore, the metabolism of 28-^{14}C-24-methylene-cholesterol was inhibited by triparanol which is a known inhibitor of dealkylation (61). The process is currently interpreted as shown in Scheme 7 and involves reversal of alkylation with scrambling of the methyl groups on C-24,25 in the positively charged transition state such that 26-^{14}C-cholesterol was obtained. The process can also be viewed as a 1,2-methyl transfer to give a 25-trimethyl transition state with equivalence and consequent scrambling of the label. If further work, especially the precise location of the label by degradation which is underway, proves that this interpretation is correct, it means that mammals fail to have 24-alkylsterols not because of the absence of an enzyme, the alkylase, but because of the control of the substrate product equilibrium, probably through cofactor control.

It has been seen that steroid biosynthesis arose very early in evolution and that the general pathway is the same throughout nature. However, various alternatives, which lead to variations in how a given steroid arises and in the structure of the final product, are both possible and actually observable.

ACKNOWLEDGMENTS

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The Biosynthesis of Cholesterol and Other Sterols by Brain Tissue: II. A Comparison of in Vitro and in Vivo Methods[1]

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ABSTRACT

When microsomal + soluble preparations of adult or 15-day-old rat brains were incubated with 2-^{14}C-mevalonic acid, ^{14}C-squalene accumulated. A metabolic block at the squalene to cholesterol stage was indicated. This prompted a comparison of all methods currently used to study cholesterol biosynthesis by brain tissue. Brain cell-free preparations from newborn, 15-day-old or adult rats accumulated ^{14}C-squalene in a similar manner, with either 2-^{14}C-acetate or 2-^{14}C-mevalonic acid as substrates. Homogenates and minced preparations from newborn or 15-day-old rats accumulated some 4,4-dimethyl sterols, but considerable conversion to free 4-desmethyl sterols (cholesterol) was evident. Sterol esters were also present in all the in vitro studies. In general, increased disruption of tissue resulted in decreased free 4-desmethyl sterol formation in vitro. Intraperitoneal injection of labeled acetate or mevalonate to newborn or 15-day-old rats produced labeled brain 4-desmethyl sterol with little accumulation of squalene or 4,4-dimethyl sterols, but the yields in brain were small compared to total amount of labeled material administered. At all ages intracerebral injection produced the best yield of labeled cholesterol for the amount of nonsaponifiable material formed.

[1]One of 12 papers being published from the Sterol Symposium, presented at the AOCS Meeting, New Orleans, April 1970.

INTRODUCTION

Brain microsomal + soluble fractions from young (15 days old) and adult rats accumulate ^{14}C-squalene when incubated with U-^{14}C-glucose, 2-^{14}C-sodium acetate or 2-^{14}C-mevalonic acid (1). This would appear to be a defect in the preparations since minced newborn brain is noted for its capacity to synthesize cholesterol (2). These results prompted a study of methods currently used for studying cholesterol biosynthesis by brain tissue.

MATERIALS AND METHODS

Animals

Rats used were purchased from National Laboratory Animal Co., St. Louis. Adult rats weighed at least 250 g. The newborn animals were 1-day-old and weighed from 5 to 7 g. The young rats were 15 days old and weighed from 30 to 35 g. Animals of both sexes were used.

Tissue Manipulations and Lipid Isolation

Minced tissue was prepared in 6 ml of 0.15 M sodium-potassium phosphate buffer (pH 7.4) per brain. The brain was repeatedly cut with scissors until of uniform consistency.

Cell-free extracts were prepared by homogenization and low speed centrifugation as previously described (3).

TABLE I

Incorporation of 2-^{14}C-Mevalonic Acid Into Nonsaponifiable and Digitonin-Precipitable Fractions of Adult Rat Brain[a]

Type of preparation	Total nonsaponifiable, dpm	Per cent of ^{14}C-incorporation	
		Nonsaponifiable	Digitonin-precipitable
Minced	21,100	7.6	<0.1
Cell-free	82,700	29.8	9.5
Microsomes + soluble	140,200	50.0	3.0

[a]Each incubation contained 0.25 μc 2-^{14}C-mevalonic acid plus tissue preparation equivalent to 1.0 g of wet brain tissue. Incubations were continued for 20 hr.

TABLE II

Incubations of 2-^{14}C-Sodium Acetate or 2-^{14}C-Mevalonic Acid With Various Types of 1-Day-Old Rat Brain Preparations

Precursor[a]	Type of preparation	Nonsaponifiable, dpm
Mevalonic acid	Minced	285,000
	Homogenate	79,400
	Cell-free	101,000
	Microsomes + soluble	41,700
Sodium acetate	Minced	20,200
	Cell-free	1,400
	Microsomes + soluble	7,400

[a]Amounts were 0.25 μc 2-^{14}C-mevalonic acid; 2.00 μc 2-^{14}C-sodium acetate. Each incubation except the microsomes + soluble contained tissue equivalent to 1.0 g of wet brain tissue. The microsomes + soluble incubations contained tissue equivalent to 0.75 g of wet brain tissue. The incubations were continued for 20 hr. Each experiment conducted in triplicate.

Homogenates were prepared in the same manner as the cell-free extracts but without the centrifugation step.

Subcellular fractions were isolated as previously described (1).

Incubation conditions and the isolation of lipid fractions were as previously described (1,3).

TABLE III

Incubations of 2-^{14}C-Sodium Acetate or 2-^{14}C-Mevalonic Acid With Various Types of 15 Day-Old Rat Brain Preparations

Precursor[a]	Type of preparation	Nonsaponifiable, dpm
Mevalonic acid	Minced	255,000
	Homogenate	60,000
	Cell-free	220,000
	Microsomes + soluble	184,000
Sodium acetate	Minced	48,200
	Cell-free	10,500
	Microsomes + soluble	34,500

[a]Amounts were 0.25 μc 2-^{14}C-mevalonic acid; 2.00 μc 2-^{14}C-sodium acetate. Each incubation except the microsomes + soluble contained tissue equivalent to 1.0 g of wet brain tissue. The microsomes + soluble incubations contained tissue equivalent to 0.5 g of wet brain tissue. The incubations were continued for 20 hr. Each experiment was conducted in triplicate.

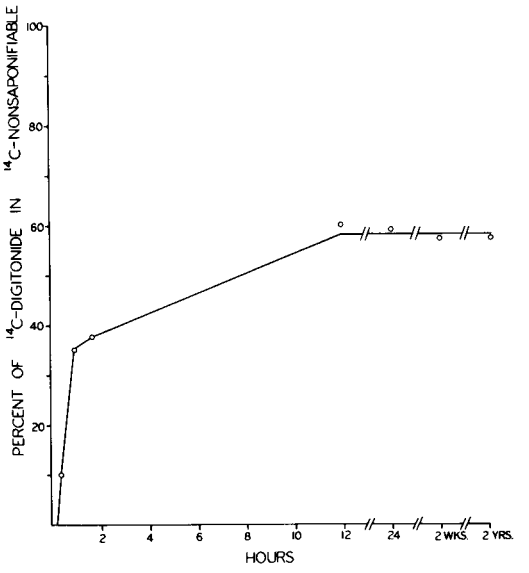

FIG. 1. Animals were injected with 0.4 μc 2-^{14}C-mevalonic acid in a volume of 0.08 ml and killed at the times indicated. After isolation and determination of the amount of ^{14}C-nonsaponifiable material present, a portion of the nonsaponifiable was taken for digitonide formation. The ratio of ^{14}C-digitonide to ^{14}C-nonsaponifiable in each time period was then determined and is shown above.

Injections

The ^{14}C-precursors injected intracerebrally or intraperitoneally were contained in an aqueous solution of 0.05 ml volume. The exact techniques used in the intracerebral injections have been described (4).

Chromatography

Nonsaponifiable fractions were applied to a 10 g alumina column with a small amount of benzene and the column eluted with 30 ml of ethanol. This eluate was taken to dryness under N_2 and redissolved in benzene for thin layer chromatography (TLC).

TLC for the separation of squalene plus sterol esters, 4,4-dimethyl sterols (C_{30} and C_{29} sterols), 4α-methyl sterols (C_{28} sterols), and 4-desmethyl sterols (C_{27} sterols, including cholesterol) was conducted on Silica Gel G. The solvent system was trimethyl pentane-ethyl acetate-acetic acid (60:30:0.6).

Where indicated the squalene and sterol ester fractions were separated on a 60 g alumina column as previously indicated (3).

Chemicals

Chemicals used in this work have been described (1).

TABLE IV

Distribution of Radioactivity in Nonsaponifiable Fractions of Brains After Incubation With 2-^{14}C-Mevalonic Acid or 2-^{14}C-Sodium Acetate[a]

Precursor	Age of animal, days	Type of preparation	Per cent of total radioactivity on TLC plate		
			Squalene and sterol esters[b]	4,4-Dimethyl and 4α-methyl sterols	4-Desmethyl sterols
2-^{14}C-Mevalonic acid	1	Minced	43.3 (18.1)	36.6	16.3
		Homogenate	21.0 (8.0)	19.6	36.1
		Cell-free	69.5 (14.6)	13.4	6.4
		Microsomes + soluble	72.9 (4.8)	10.6	5.4
2-^{14}C-Mevalonic acid	15	Minced	33.8 (21.6)	43.1	21.0
		Homogenate	29.2 (20.5)	30.8	24.5
		Cell-free	32.1 (22.3)	31.9	17.6
2-^{14}C-Sodium acetate	1	Minced	24.4 (10.2)	36.8	17.0
		Cell-free	73.9 (14.8)	21.3	2.4
		Microsomes + soluble	56.2 (3.7)	27.5	5.4
2-^{14}C-Sodium acetate	15	Minced	13.2 (8.8)	26.7	39.3
		Cell-free	56.2 (38.8)	30.5	6.2

[a]Material chromatographed is that previously indicated in Tables II and III.

[b]Number in parentheses indicates the portion of the total TLC radioactivity that was esterified sterol.

Radioactivity

Radioactivity was determined using an Ansitron scintillation spectrometer as described (3).

RESULTS

In Vitro

Metabolism of 2-^{14}C-Mevalonic Acid by Various Adult Brain Preparations. A comparison of the incorporation of 2-^{14}C-mevalonic acid into nonsaponifiable material by adult brain preparations, and the portion of this precipitated by digitonin, is shown in Table I. There was a marked increase in ^{14}C-nonsaponifiable biosynthesis in the microsomal + soluble preparations as compared to the minced, with the cell-free preparation intermediate in biosynthetic capacity. However, the ^{14}C-sterol produced (material precipitated by digitonin) was considerably diminished in the microsomal + soluble preparation.

Newborn Rats: Incorporation of Labeled Cholesterol Precursors Into ^{14}C-Nonsaponifiable Fractions In Vitro. Data showing the incorporation of 2-^{14}-C-sodium acetate and 2-^{14}C-mevalonic acid into nonsaponifiable fractions of minced tissue, simple homogenates, cell-free, and microsomal + soluble preparations of newborn rat brains are presented in Table II. The minced brain preparation, containing 2-^{14}C-mevalonic acid, has twice the labeled nonsaponifiable material as any other experiment shown. The minced preparation containing 2-^{14}C-sodium acetate was also far

TABLE V

Utilization of Injected 2-^{14}C-Sodium Acetate or 2-^{14}C-Mevalonic Acid by 1 Day Old Rats

Precursor[a]	Type of experiment	Nonsaponifiable, dpm/g wet wt of brain		
		Brain	Liver	Carcass
Mevalonic acid	Intracerebral	28,100	1,730	17,100
	Intraperitoneal	370	6,240	21,600
Sodium acetate	Intracerebral	24,000	1,050	15,300
	Intraperitoneal	150	2,300	105,500

[a]Amounts were 0.25 μc 2-^{14}C-mevalonic acid; 2.50 μc 2-^{14}C sodium acetate. Animals were killed 20 hr after injection. Dpm of liver and carcass are expressed as quotients of the total gram wet weight of brain per experiment. Each experiment was conducted in triplicate.

TABLE VI

Utilization of Injected 2-^{14}C-Sodium Acetate or 2-^{14}C-Mevalonic Acid by 15-Day-Old Rats

Precursor[a]	Incubation period, hr	Type of experiment	Nonsaponifiable, dpm/g wet wt of brain		
			Brain	Liver	Carcass
Mevalonic acid	5	Intracerebral	125,000	3,800	6,850
	20		61,500	680	24,900
	5	Intraperitoneal	8,160	39,300	57,300
	20.		940	14,300	107,000
Sodium acetate	5	Intracerebral	64,700	115	7,480
	20		32,200	410	32,500
	5	Intraperitoneal	17,940	2,990	21,300
	20		19,000	390	18,600

[a]Amounts were 0.25 μc 2-^{14}C-mevalonic acid; 2.50 μc 2-^{14}C-sodium acetate. Dpm of liver and carcass are expressed as quotients of the total gram wet weight of brain per experiment. Each experiment was conducted in triplicate.

superior to the other types of 2-^{14}C-sodium acetate-containing preparations in amount of ^{14}C-nonsaponifiable formed.

15-Day-Old Rats: Incorporation of Labeled Cholesterol Precursors Into ^{14}C-Nonsaponifiable Fractions In Vitro. Data showing the incorporation of 2-^{14}C-sodium acetate and 2-^{14}C-mevalonic acid into nonsaponifiable fractions of minced tissue, simple homogenates, cell-free, and microsomal + soluble preparations of 15-day-old rat brains are given in Table III. Here the minced preparation has not outstripped the other incubations in amounts of labeled nonsaponifiable formed; the 2-^{14}C-mevalonic acid incubations with cell-free and subcellular preparations yielded as much ^{14}C-nonsaponifiable material as the minced preparation. The 2-^{14}C-sodium acetate minced incubation was still slightly superior to the other preparations containing 2-^{14}C-sodium acetate in amount of ^{14}C-nonsaponifiable formed, but not nearly so great as with the 1-day-old 2-^{14}C-sodium acetate incubations.

Nature of ^{14}C-Nonsaponifiable Material From Newborn and 15-Day-Old Rat In Vitro Experiments. ^{14}C-Nonsaponifiable fractions obtained from incubated minced tissue homogenates, cell-free and subcellular preparations of newborn and 15-day-old rat brain were subjected to TLC fractionation and the resulting data are presented in Table IV. Only the homogenates and minced tissue preparations synthesized an appreciable amount of free 4-desmethyl sterol. Both newborn and 15-day-old

TABLE VII

Distribution of Radioactivity in Nonsaponifiable Fractions of Brain and Liver of 1-Day-Old Rats After Injection of 2-^{14}C-Mevalonic Acid or 2-^{14}C-Sodium Acetate[a]

Precursor	Type of injection	Tissue	Per cent of total radioactivity of TLC plate		
			Squalene and sterol esters[b]	4,4-Dimethyl and 4α-methyl sterols	4-Desmethyl sterols
2-^{14}C-Mevalonic acid	Intracerebral	Brain	2.7	5.8	90.6
		Liver	6.1 (1.1)	2.6	90.7
	Intraperitoneal	Brain	1.1	2.6	95.7
		Liver	8.4 (2.1)	7.6	83.0
2-^{14}C-Sodium acetate	Intracerebral	Brain	1.0	2.8	94.8
		Liver	3.3	8.0	88.4
	Intraperitoneal	Brain[c]	---	---	---
		Liver	1.0	1.1	96.8

[a]Material chromatographed is that previously indicated in Table V.

[b]Number in parentheses indicates the portion of the total TLC radioactivity that was esterified sterol.

[c]Insufficient radioactivity for a TLC determination.

TABLE VIII

Distribution of Radioactivity in Nonsaponifiable Fractions of Brain and Liver of 15-Day-Old Rats After Injection of 2-^{14}C-Mevalonic Acid or 2-^{14}C-Sodium Acetate[a]

Precursor	Period of injection, hr	Type of injection	Tissue	Per cent of total radioactivity on TLC plate		
				Squalene and sterol esters[b]	4,4-Dimethyl and 4α-methyl sterols	4-Desmethyl sterol
2-^{14}C-Mevalonic acid	5	Intracerebral	Brain	0.4	20.5	77.7
			Liver	65.7	11.3	33.2
	5	Intraperitoneal	Brain	2.1	18.7	78.6
			Liver	26.1	12.9	60.6
	20	Intracerebral	Brain	5.8	12.8	81.0
			Liver	28.1 (5.0)	5.0	67.0
	20	Intraperitoneal	Brain[c]	---	---	---
			Liver	25.3 (3.5)	7.4	66.6
2-^{14}C-Sodium acetate	5	Intracerebral	Brain	1.5	12.1	84.3
			Liver	26.4	12.7	58.8
	5	Intraperitoneal	Brain	0.7	11.3	85.5
			Liver	27.0	9.1	60.9
	20	Intracerebral	Brain	0.5	2.2	97.0
			Liver	7.7 (1.1)	1.3	89.7
	20	Intraperitoneal	Brain	0.1	2.4	97.2
			Liver	15.2 (2.2)	4.1	76.1

[a]Material chromatographed is that previously indicated in Table VI.

[b]Number in parentheses indicates the portion of the total TLC radioactivity that was esterified sterol.

[c]Insufficient radioactivity for a TLC determination.

tissue were equally active in this respect. With the exception of the microsomal + soluble incubations, the in vitro incubations produced considerable sterol ester. Examination of several ester fractions after saponification indicated that 50% or less of the esterified sterol was 4-desmethyl sterol. The remainder of the esterified sterol was a mixture of 4α-methyl and 4,4-dimethyl sterol.

These results with newborn tissue document the work of Srere et al. (2), although a precise comparison cannot be made since these investigators determined "cholesterol" only as material precipitated by digitonin.

In Vivo

Intracerebral Injection of 2-^{14}C-Mevalonic Acid Into Adult Rats. When 2-^{14}C-mevalonic acid was injected intracerebrally the uptake into digitonin-precipitable material was twice as high as any of the in vitro experiments shown in Table I. This is shown in Figure 1. Within 12 hr after injection the amount of material precipitable with digitonin had reached its maximum of approximately 60% of the total nonsaponifiable. This percentage was shown to be constant for up to two years after injection.

Newborn Rats: Quantitative Aspects of Intraperitoneal and Intracerebral Injection of Cholesterol Precursors. Since either intraperitoneal or intracerebral injection of labeled substances involve distribution of radioactivity to other parts of the body besides the brain, it was of interest to determine the distribution of radioactivity after such injections. Data for such an experiment with newborn rats are given in Table V. It will be noted that after intracerebral injection, not unexpectedly, most of the counts in the nonsaponifiable fraction were in the brain. After intraperitoneal injection much more radioactivity was present in liver than in brain. Following either type of injection a considerable amount of radioactivity was found in the carcass. In the case of the intraperitoneal injection of 2-^{14}C-sodium acetate the amount of label present in the brain and liver was insignificant compared to that present in the carcass. At 1 day the penetration of both precursors into the brain and utilization for brain sterol biosynthesis after intraperitoneal administration was poor.

15-Day-Old Rats: Quantitative Aspects of Intraperitoneal and Intracerebral Injection of Cholesterol Precursors. Data exploring the same aspects discussed in the previous section, but using 15-day-old rats, are given in Tabel VI. After intraperitoneal injection the distribution of counts in brain, liver and carcass are in general the same for this age rat as for newborn animals, with two exceptions. The intraperitoneal injections of 2-^{14}C-sodium acetate for 5 and 20 hr duration both indicate that the brain is quite permeable to acetate, for a large amount of the total label in the animal was in the brain. The opposite is true of the intraperitoneal injections of mevalonic acid; here a much smaller percentage of the total label is present in the brain. At 15 days of age apparently acetate is more readily taken up by the brain and metabolized to sterol than is mevalonic acid.

The Nature of ^{14}C-Nonsaponifiable Material Following Intraperitoneal and Intracerebral Injection of Labeled Cholesterol Precursors Into Newborn and 15-Day-Old Rats. Nonsaponifiable fractions of brain and liver, following intraperitoneal or intracerebral injection of 2-^{14}C-sodium acetate or 2-^{14}C-mevalonic acid, were subjected to TLC, following preliminary fractionation on an alumina column. Counts in the squalene and ester fractions, 4,4-dimethyl and 4α-methyl sterol fraction, and 4-desmethyl sterol fractions were determined. These data are shown in Table VII for newborn and Table VIII for 15-day-old rats. The incorporation into 4-desmethyl sterol was greater than 80% in both brain and liver of newborn rats following either intracerebral or intraperitoneal injection of the labeled precursors. This is reflected in the low percentage of counts in the squalene and 4,4-dimethyl sterol (lanosterol) regions, these having undergone turnover. The results with the 15-day-old brain were approximately the same as with the newborn rats. Apparently up to at least 15 days of age there is no pronounced preference for either acetate or mevalonic acid once it is in the brain.

In comparison, the livers incorporated the labeled materials much more slowly than the brains of the 15-day-old animals. After 5 hr the 2-^{14}C-sodium acetate was slightly better utilized than the 2-^{14}C-mevalonic acid, in terms of per cent of free 4-desmethyl material present. Twenty hours after injection the free 4-desmethyl sterol present in the liver was still less than that of the brain of the same animal, but the percentage of free 4-desmethyl sterol in both liver and brain were increased. The presence of a small amount of esterified sterol was also indicated in the liver nonsaponifiables.

DISCUSSION

In Vitro Biosynthesis

In the newborn and 15-day-old rat, the use of homogenates and minced brain tissue is reasonably satisfactory, but this method only partially indicates the potential of the

immature rat brain to synthesize sterol. With partial or complete fractionation of the brain tissue, as in preparation of cell-free extracts or subcellular preparations, a marked loss in the capacity to synthesize cholesterol results, with brains from animals of any age. With minced tissue from adult rat brain, where some cellular integrity is maintained, the problem seems to involve the inability of precursors such as acetate or mevalonate to penetrate to the biosynthetic site. This is evident by the fact that adult cell-free preparations exhibit considerably more conversion to nonsaponifiable material than do minced preparations. Galli et al. (5) have indicated that a considerable portion of the nonsaponifiable fraction resulting from 2 hr incubation of 20 μc of 2-^{14}C-mevalonic acid with a minced preparation of adult human brain was labeled sterol; however, the amount of utilizable mevalonate incorporated was only on the order of 0.20%.

It has been clearly shown in the preceding manuscript, however, that the principal defect in isolated preparations of brain, young or old, is a blockage at the squalene to cholesterol stage, and more probably at the squalene to desmosterol stage. It has not yet been ascertained whether this is due to an instability of the enzymes involved or to inhibitory factors present in brain tissue released or made more accessible on disruption of the tissue. The defect is believed to be mainly microsomal in origin.

Others have reported observations on cholesterol biosynthesis by brain homogenates of young animals but no direct comparison can be made with our data. For example, Grossi et al. (6) and Garattini et al. (7) used nicotinamide in their homogenates. We have found nicotinamide inhibitory to sterol formation in adult brain cell-free extracts (3). Most reported incubations of brain homogenates used for cholesterol biosynthetic studies of brain have been carried out for 2 to 3 hr. We have found that a minimum of 4 hr is required for maximum sterol biosynthesis by most types of brain preparations and a minimum of 10 hr in the case of adult brain preparations (1). Also in vivo it seems that a minimum of 4 or 5 hr is necessary before a major portion of the label, 2-^{14}C-sodium acetate or 2-^{14}C-mevalonic acid is turned over to sterol. The results of Garattini et al. (7,8) showed that after 2 hr of incubation young brain in vitro and in vivo incorporated acetate into sterols more readily than did mevalonate. There is no question that the brevity of the incubations was a factor in these results; however, it was shown in a later paper than when 12-day-old rat brain tissue slices were incubated for up to 4 hr with the potassium salt of 2-^{14}C-mevalonic acid, the incorporation was slightly better than with 2-^{14}C-acetate (9). Mevalonolactone, which had been used in prior experiments, was found to be a poor precursor of sterols in brain. In 2 hr incubations of cell-free extracts of 12-day-old brain, potassium mevalonate was found to give approximately twice as much digitonin-precipitable material as 2-^{14}C-acetate. Again, however, the amounts of digitonin-precipitable material formed were low compared to the amount of sterol found in the TLC of the cell-free incubations of our work. Two factors could be that not only were the incubations for only 2 hr, but they also contained nicotinamide.

In Vivo Biosynthesis

The data presented in this and the preceding manuscript demonstrate that only intracerebral injection of labeled cholesterol precursors can indicate the true capacity of brain to synthesize cholesterol, at least at our present stage of knowledge. This applies to animals of any age. Intraperitoneal injection appears to be a satisfactory method for certain precursors, but for reasons evaluated in detail some years ago (10), is not entirely adequate for the most effective labeling of sterol in the adult animal. Limited labeling of sterols of adult rat brain has been achieved by Weiss et al. (11) using massive doses of 2-^{14}C-mevalonate injected intravenously. Kabara has achieved significant labeling of brain sterols in mice by means of intraperitoneally injected labeled acetate, glucose and leucine as precursors (12-15). Similar labeling of adult brain sterol with glucose has been shown by Moser and Karnovsky (16) and Smith (17,18).

Recently Chevallier and Cautheron have found that the sterols of adult rat brain were well labeled by means of intracisternal or intraventricular injection of 2-^{14}C-mevalonic lactone (19). They have shown that these methods give a sufficient amount of labeling so that radioautographic studies of the labeled sterols may be done on slices from these brains (20).

As indicated in this manuscript we have found no basic difference between the behavior of acetate or mevalonate except the expected quantitative difference due to mevalonate being more directly on the isoprenoid biosynthetic pathway.

ACKNOWLEDGMENTS

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Preparation of Tritium-Labeled Sterols and the Synthesis of Labeled-24-Azacholesterol[1]

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ABSTRACT

A method is presented for the preparation of 2,4-^{3}H-sterols (stigmasterol, β-sitosterol, campesterol, fucosterol and 7-dehydrocholesterol) via the technique of exchange-labeling of keto steroids and their subsequent conversion to sterols. The preparation of tritium-labeled 3β-hydroxy-24-norchol-5-en-23-oic acid and the synthesis of 2,4-^{3}H-24-azacholesterol are reported. Some advantages of the exchange-labeling method over the conventional catalytic exchange method are discussed.

INTRODUCTION

Klein and Knight (1) showed that keto steroids were exchange-labeled by column chromatography on basic alumina that contains tritiated water (HTO). Further studies (2) established that this exchange-labeling involved mainly the enolic hydrogens adjacent to the keto group and that essentially complete exchange occurred during a single passage of the compound through the alumina column. Subsequently, the tritium-labeled keto steroids could be reduced by metallic hydrides to the corresponding alcohols without appreciable loss of label; the tritium of these alcohols was no longer exchangeable. This technique of exchange-labeling provides an expeditious, convenient and efficient method of preparing high specific activity compounds of high purity, and the method has been successfully utilized in the preparation of 2,4-^{3}H-14α-methylcholest-7-en-3β-ol (3) and 2,4-^{3}H-labeled bile acids (4).

In the present paper, we extend and further demonstrate the practical application of exchange-labeling and report the preparation of highly purified 2,4-^{3}H-sterols (stigmasterol, β-sitosterol, campesterol, fucosterol and 7-dehydrocholesterol). The preparation of the tritium-labeled Δ^5-sterols involves the sequence of reactions shown in the scheme for the labeling of β-sitosterol (I), namely: the oxidation of the sterol to the 4-en-3-one compound (II); exchange label of II on alumina to III; and the conversion of III to the enol-acetate (IV); and the reduction of IV with sodium borohydride to give the 2,4-^{3}H-epimers V and VI. We also prepared the tritium-labeled 3β-hydroxy-24-norchol-5-en-23-oic acid (VIIa) by this process, and synthesized the 2,4-^{3}H-24-azacholesterol (XI) from this hydroxy acid.

EXPERIMENTAL PROCEDURES

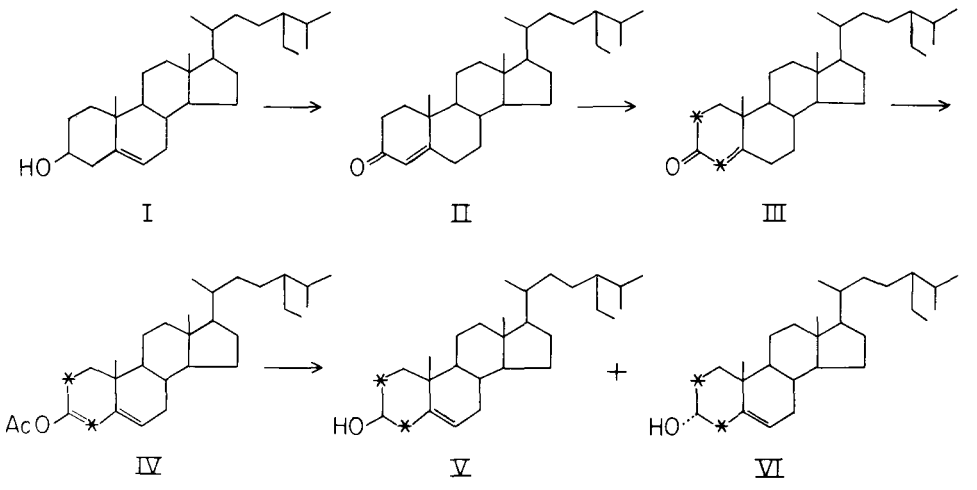
Instrumentation

Melting points were observed on a Kofler block; UV spectra were obtained with a Bausch and Lomb spectrophotometer 505; and IR spectra were obtained with a Perkin-Elmer model 221 prism-grating spectrophotometer. Gas liquid chromatographic (GLC) analyses were made on a Barber-Colman model 10 chromatograph; the GLC system was 0.75% SE-30 coated on Gas-Chrom P. Measurements of radioactivity were made with a Packard Tri-Carb Liquid Scintillation Spectrometer.

Materials

Basic and neutral alumina (Woelm) were obtained from Alupharm Chemicals, New Orleans, and the tritiated water (HTO) was applied to the basic alumina as previously described (4). Thin layer chromatographic (TLC) analyses were made on chromatoplates prepared with Silica Gel G (Brinkmann Instruments Inc., Westbury, N.Y.). The stigmasterol was a gift of the Upjohn Company, Kalamazoo, Michigan, and the β-sitosterol (>99% purity) was prepared from stigmasterol by the method of Steele and Mosettig (5). The campesterol (>98% purity) was obtained by fractional crystallization from acetone of soybean sterols from which the stigmasterol had been removed. The fucosterol was a gift from Glenn Patterson of the University of Maryland. The 7-dehydrocholesterol was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio. The 3β-hydroxy-24-norchol-5-en-23-oic acid was prepared as previously reported (6). The 3-keto steroids, for example, II, were prepared by an Oppenauer oxidation of the respective sterol.

[1]One of 12 papers to be published from the "Sterol Symposium" presented at the AOCS Meeting, New Orleans, April 1970.

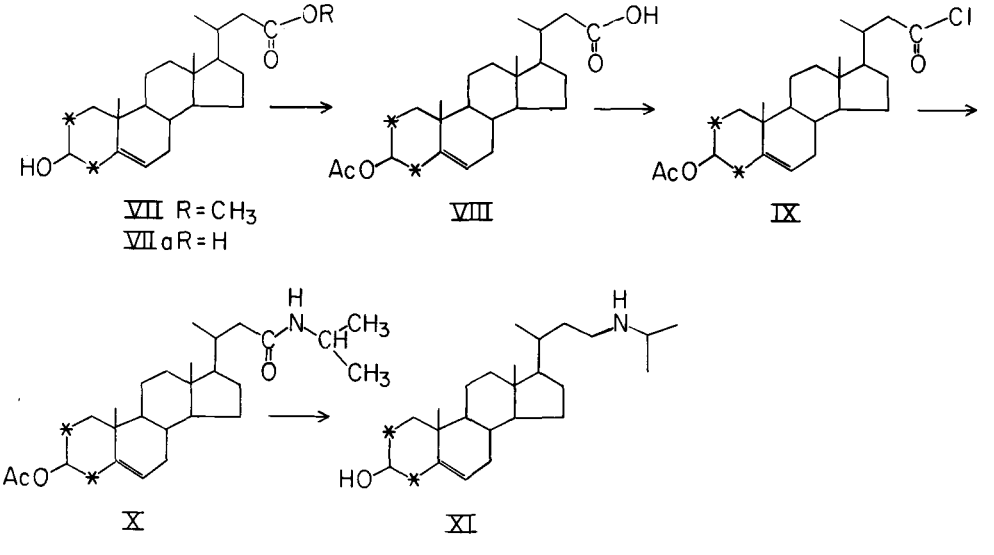


SCHEME I

The 4-en-3-keto compounds were chromatographed over neutral alumina (activity grade II) and the fractions were analyzed by TLC and IR spectroscopy and then recrystallized. The methyl 3β-hydroxy-24-norchol-5-en-23-oate did not readily undergo an Oppenauer oxidation, thus the methyl 3-one-24-norchol-4-en-23-oate was prepared by the rapid oxidation of the methyl ester in acetone with a solution of chromic acid in dilute sulfuric acid (7) followed by an isomerization of any remaining 5-en-3-one compound to the 4-en-3-one compound with oxalic acid in ethanol (8). The final product was purified by column chromatography and recrystallization.

General Procedure for the Preparation of 2,4-^{3}H-Sterols [β-Sitosterol (I) as Model Compound]

About 300 mg of stigmast-4-en-3-one (II) was exchange-labeled by passage through a column of basic alumina as previously reported (4). The tritium-labeled unsaturated ketone (III), 6 ml of isopropenyl acetate, and 0.02 ml of concentrated sulfuric acid were refluxed for 2 hr (9). To the mixture was added 100 mg of anhydrous sodium acetate, and the solvent was removed in vacuum. The residue was triturated with chloroform, and the chloroform was separated and removed in vacuum. An IR spectrum of the residue taken in chloroform showed no α,β-unsaturated carbonyl. A solution of 400 mg



SCHEME II

TABLE I

Radiochemical Purity and Tritium Content of Final Products

Compound	Mp, C	Yield,[a] %	Specific activity cpm/μg X 10^3	Radiochemical purity, % Analyses TLC	GLC
β-Sitosterol	139.5-140	65	57	99.5	94
Campesterol	160.5-161.5	66	67	99.8	93
Stigmasterol	170-171	55	90	99.3	93.5
7-Dehydrocholesterol	140-142	25	44	97.5	93
Fucosterol	124-126	40	25.4	99	95.2
Methyl 3β-hydroxy-24-norchol 5-en-23-oate	143-144	50	13	99.5	93
3β-Hydroxy-24-norchol-5-en-23-oic acid	232-234	44	12.5	99	---
24-Azacholesterol	164.5-166	70[b]	11.9	98.8	95.6

[a]Purified yield of the 3β-epimer calculated from the 4-en-3-one compound.
[b]Overall purified yield calculated from the hydroxy-acid (VIIa).

of sodium borohydride in 15 ml of water and 35 ml of 95% ethanol was added to a solution of the crude enol-acetate (IV) in 100 ml of 95% ethanol at 5 C (10) and the reaction mixture was stirred at 5 C for 2 hr and then left overnight at 5 C. The mixture was then heated to boiling and 1.0 g of solid potassium hydroxide was added. Most of the solvent was removed in vacuum, the solution was diluted with water and the precipitate was collected. The precipitate in 70 ml of ethanol that contained 0.2 ml of concentrated hydrochloric acid solution was refluxed for 2 hr. (this converts any 3-hydroxy-4-en compounds to unsaturated hydrocarbons). The solution was reduced in volume in vacuum and diluted with water, and the precipitate was collected and dried. The material was then chromatographed over 30 g of neutral alumina (activity grade II), and the following fractions were collected: 1, 200 ml fraction of hexane; 2, 200 ml fractions of hexane-benzene (1:1); 3-6, 100 ml fractions of benzene; and 7-8, 100 ml fractions of ether. The fractions were analyzed by TLC (the 3α- and the 3β-epimers were inseparable by GLC on SE-30). Fractions 3-4 yielded 45 mg of the 3α-epimer (VI). Fraction 6-7 combined and recrystallized from dilute methanol gave 181 mg of 2,4-^{3}H-β-sitosterol (V), mp 139.5-140 C; a second crop of 10 mg of crystals was also collected, mp 139-140 C.

Methyl 2,4-^{3}H-3β-hydroxy-24-norchol-5-en-23-oate (VII). The tritium-labeled methyl 3β-hydroxy-24-norchol-5-en-23-oate was prepared in a manner similar to that used to prepare tritium-labeled β-sitosterol except that when the enol acetate-acid methyl ester had been reduced with sodium borohydride in ethanol, it was necessary to convert the ester to the free acid and then remethylate with diazomethane (if this was not done a mixture of the methyl and ethyl ester resulted). Tritium-labeled methyl ester (VII), separated from its 3β-epimer by preparative TLC on Silica Gel H plates, in a solvent system of benzene-ethyl acetate (6:1) gave, after crystallization from hexane, 110 mg of pure VII, mp 143-144 C; literature (6), mp 143-144 C.

2,4-^{3}H-3β-Hydroxy-24-norchol-5-en-23-oic acid (VIIa). A mixture of 108 mg of the methyl ester (VII), 75 ml of methanol and 4.0 g of potassium hydroxide was refluxed for 3 hr and then about 75% of the solvent was removed in vacuum. The mixture was diluted with a small quantity of water, the solution was acidified with a dilute solution of hydrochloric acid, and the precipitate was collected. Recrystallization of the material from ethyl acetate gave 92 mg of the tritium-labeled acid (VIIa), mp 232-234 C; literature (6), mp 228-232 C.

2,4-^{3}H-24-Azacholesterol (XI). A mixture of 80 mg of VIIa was acetylated by the pyridine-acetic anhydride method to give the acetate-acid (VIII) in quantitative yield. Compound VIII, 10 ml of hexane, 5 ml of benzene and 1 ml of thionyl chloride were refluxed overnight. The mixture was then concentrated to dryness in vacuum to give the crystalline acid chloride (IX). The acid chloride in 10 ml of benzene was added to a solution of 20 ml of benzene and 2 ml of isopropylamine at 10 C. The mixture stood at room temperature for 5 hr and was then concentrated to dryness in vacuum. A TLC analysis of the crude ^{3}H-acetate-amide (X)

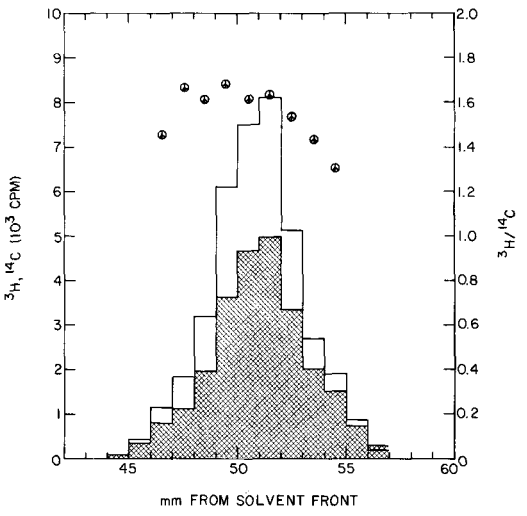


FIG. 1. TLC of methyl 2,4-^{3}H-3β-hydroxy-24-norchol-5-en-23-oate acetate (outer line) with methyl 3β-hydroxy-24-norchol-5-en-23-oate 1-^{14}C acetate (cross hatched area). Circles: isotope ratio.

showed only one zone, and it had an Rf value identical to that of unlabeled compound (X). The acetate-amide in 20 ml of dry tetrahydrofuran was added to 30 ml of dry tetrahydrofuran that contained 0.5 g of lithium aluminium hydride, and the mixture was refluxed overnight. The excess hydride was decomposed by the dropwise addition of ethyl acetate, water and the solution was treated with 20 ml of water and 4.0 g of sodium hydroxide. The organic layer was separated by decantation and was concentrated to dryness in vacuum. The residue, recrystallized twice from dilute acetone containing a trace of pyridine, gave 60 mg of the tritium-labeled 24-azacholesterol (XI), mp 164.5-166 C; literature (11), mp 164-166 C.

RESULTS AND DISCUSSION

Previous preparations of labeled steroids and sterols by exchange-labeled chromatography (1-4) have not included the synthesis of ^{3}H-Δ^{5}-sterols because the required intermediates, Δ^{5}-3-keto steroids, are rapidly isomerized on basic alumina to Δ^{4}-3-keto steroids from which Δ^{5}-sterols are not regenerated by reduction with metallic hydrides. The conversion of the labeled Δ^{4}-3-keto steroids to the enol-acetates and their subsequent reduction to Δ^{5}-sterols by the procedures described is an alternative which has been used to prepare 4-^{14}C-cholesterol (9,10).

The results given in Table I demonstrate the practicability of using these reactions following the exchange-labeling process to prepare tritium-labeled sterols of high purity and specific activity. The purified yields of the tritiated sterols are reasonably good, even though 15-20% of the 3α-epimers are formed at the terminal step of the process. However, the 3α-epimer can be converted to the 3β-sterol without loss of specific activity simply by repeating the sequence of reactions shown in the scheme and excluding the exchange-labeling step. The 3β- and 3α-epimers were readily separated by column chromatography and TLC, and the results of TLC analyses showed that the radiochemical purity of the prepared compounds was >97% in all cases. Although the epimers were not separable by GLC on an SE-30 column, GLC analyses were made to determine whether impurities not detected by TLC were present. The melting points and IR spectra of the ^{3}H-sterols were identical to those of the respective unlabeled compounds.

Further evidence of the radiochemical purity of the preparations was obtained by comparing the tritium-labeled sterol to the original starting material by dual-label chromatography. The ^{14}C-acetates of the starting products were prepared by acetylation with ^{14}C-acetic anhydride while the tritium-labeled sterols were acetylated with unlabeled acetic anhydride. Each pair of original and labeled sterols was subjected to TLC, and the developed chromatograms were sectioned in 1 mm zones for liquid scintillation counting, using the TLC scraper of Snyder (12). A representative chromatogram that illustrates the purity of the methyl 3β-hydroxy-24-norchol-5-en-23-oate is shown in Figure 1. The isotope ratio and peak dispersions were calculated from the values for ^{14}C and ^{3}H in each segment of the peak. In all instances, the dispersion of the tritium-labeled sterol acetate was equal to or smaller than that of the radiocarbon-labeled form, indicating that the former compound was as pure as (and in most cases, purer than) the starting material (13).

The successful preparation of ^{3}H-7-dehydrocholesterol was entirely unexpected since the intermediate cholesta-4,7-dien-3-one appeared to react and decompose on the alumina column. Yet, we were able to obtain pure ^{3}H-7-dehydrocholesterol in an overall 25% yield. We are confident that if proper precaution and care are taken in carrying out the preparation the yield of ^{3}H-7-dehydrocholesterol can be improved.

The synthesis of the ^{3}H-24-azacholesterol from the ^{3}H-hydroxy-acid (VIIa) through a four step synthesis with essentially no loss in specific activity further illustrates the stability of the tritium in these compounds.

The specific activity of 12,000-90,000

cpm/μg shown for the compounds listed in Table I is 40-300 times greater than the 300 cpm/μg required for most of our metabolic studies concerned with the utilization of sterols and the mechanism of dealkylation of phytosterols and their conversion to cholesterol in insects. This high specific activity and the ease of preparation should facilitate and expedite metabolic studies of these naturally occurring phytosterols in other invertebrates and vertebrates.

The labeling method has many advantages over conventional catalytic exchange-labeling techniques. It is rapid and both milligram and gram quantities of compounds can be rapidly prepared; it is more economical, and the products are obtained in high yield and have excellent radiochemical purity. In contrast, we have had difficulty or have been completely unsuccessful in purifying ^{3}H-sterols prepared commercially by the catalytic exchange method. Another advantage over random labeling is that the positions of labeling are known, which permits the specific activity of a metabolite to be predicted more accurately. For example, during the conversion of commercially prepared randomly ^{3}H-labeled β-sitosterol to cholesterol in an insect, the specific activity of the cholesterol was considerably less than that of the β-sitosterol, and part of this reduction in specific activity was caused by the loss of the highly-labeled C-24 substituent of the β-sitosterol during the dealkylation process. However, ^{3}H-stigmasterol prepared by the method presented has been converted to ^{3}H-22-*trans*-cholesta-5,22-dien-3β-ol with essentially no change in specific activity based on that expected from the ^{3}H-stigmasterol (14). Thus, the advantages of preparing synthetic ^{3}H-compounds with tritium at known positions are quite considerable.

The 3β-hydroxy-24-norchol-5-en-23-oic acid has been shown to block the conversion of β-sitosterol to cholesterol and to bring about an accumulation of desmosterol in the tobacco hornworm, *Manduca sexta* (Johannson) (6). This acid also interferes with the Δ^{24}-reductase enzyme system of the rat and brings about an accumulation of desmosterol in its serum and liver (15). The 24-azacholesterol, in addition to other effects, has similar activity in the tobacco hornworm (16). We have prepared the ^{3}H-hydroxy-acid (VIIa) and used it to prepare ^{3}H-24-azacholesterol and these two ^{3}H-compounds will be used in studies concerned with the mode of action of these inhibitors in insects. The synthesis of ^{3}H-24-azacholesterol demonstrates that many of the potent hypocholesterolemic azasterols can be prepared as the 2,4-^{3}H-labeled forms which should enhance future studies concerned with their metabolism and mode of action in vertebrates and invertebrates.

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Introduction to the Symposium on Chemistry and Biochemistry of Tocopherols[1]

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It has been said that the way to grow old gracefully is to stay young in heart. Perhaps this applies to vitamin E. Almost 50 years have passed since Evans and Bishop recognized the existence of an unidentified nutrient necessary for reproduction in rats (1). During the last 20 years, about 400 papers on vitamin E have been published annually. Yet the puzzle of vitamin E remains as fresh and challenging as ever.

There is no question, however, that vitamin E is maturing. The Food and Nutrition Board recognized its essentiality for man in 1964 and tabulated Recommended Dietary Allowances in 1968 (2). Recognition of the need does not necessarily provide practical guidance for fulfilling the need. Current information is required to establish dietary intakes and relationships to other nutrients such as polyunsaturated fatty acids and protein. Clinical or biochemical methods are needed to detect marginal or imminent deficiency in man. An important step in the latter direction has been taken by Grimes and Leonard (3), who have shown that tissue levels of α-tocopherol correlate positively with plasma levels of 0.5 mg% or below.

The development of analytical methods employing paper, column, thin layer or gas liquid chromatography (GLC) has afforded much more sensitive and specific means for determining tocopherol contents. Within the last decade, the group of naturally occurring compounds possessing vitamin E activity was shown to be comprised of α-, β-, γ- and δ-tocopherols and their analogous tocotrienols containing three unsaturated bonds in the side chain (4). More precise information on the biological activity of these compounds and increased application of these methods to foods and feedstuffs are both needed.

The role of vitamin E in the nutrition of domestic animals has not been neglected. Here too, however, advances in our knowledge have raised new questions. Perhaps the most intriguing has been the relationship between vitamin E and selenium. Although the need for selenium as an essential trace element in its own right is increasingly accepted (5), the interplay between these two nutrients is poorly understood.

Nor has the role of tocopherols in foods and food technology been ignored. The development of new foods as substitutes for traditional foods and the continuing exploration of new methods of preservation such as radiation raise questions about alterations in the intake of vitamin E. The catalysis of linoleic acid oxidation by proteins containing heme (6) may well be influenced by tocopherols. Although not the best antioxidant per se, α-tocopherol is uniquely suited for its role as a biological antioxidant because it is absorbed and deposited in body tissues.

This symposium is divided into two parts with the realization that this is simply a convenient way to separate one package. The morning session is devoted to the "Chemistry of Tocopherols". W.A. Skinner has been interested in reaction products of the tocopherols for several years and has speculated on the intermediate free radicals and their role (7). Dimers and trimers of tocopherols, to be discussed by B.S. Strauch, are no longer oddities of chemistry but are realities of mammalian biochemistry and have been reported to occur in vegetable oils (8). R.H. Bunnell and his coworkers have made extensive contributions to the various chromatographic techniques for tocopherols and can provide guidelines to the preferred method. H.T. Slover, whose analytical specialty is GLC, will devote most of his report to tocopherol contents of foods and fats as determined by the more reliable methods now available.

The afternoon session brings together five topics of current interest in the "Biochemistry of Tocopherols". One of J.G. Bieri's major interests has been delineating the real meaning of vitamin E and its relationship to other compounds which appear to replace, spare or interact with tocopherols. S.R. Ames' extensive contributions in this area have included assessment of the biological activity of 2L-α-tocopherol made available by resolution of the epimeric mixture synthesized from natural phytol (9). The question of the in vivo role and disposition of α-tocopherol has flowed and ebbed like the tides. P.B. McCay's work starts a new chapter with a fresh viewpoint. R. Carolla's work recalls

[1]One of six papers to be published from the Symposium "Chemistry and Biochemistry of Tocopherols" presented at the ISF-AOCS World Congress, Chicago, September 1970.

the story of retrolental fibroplasia (10) but is more pertinent now to space capsule environments and to the increasing utilization of hyperbaric oxygenation for medical-surgical purposes. Finally, all of these topics may well be relevant to A.L. Tappel's interest in the interplay of antioxidants in the body, with particular emphasis on vitamin E.

This is a panoramic view of the symposium program. I hope that you will find it as interesting and exciting as I expect it to be.

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Reaction Products of Tocopherols[1]

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ABSTRACT

Tocopherols readily undergo oxidation with a variety of oxidizing agents. Considerable effort has gone into isolation and identification of these various oxidation products. In many cases they can undergo further transformations upon treatment with various chemical reagents. This review will focus on the oxidation of α-tocopherol and transformation of its oxidation products to new derivatives. Dimeric and trimeric oxidation products will not be covered. Early work on the oxidation of α-tocopherol led to identification of α-tocopherol quinone as an oxidation product formed by $FeCl_3$ oxidation. Stronger oxidizing conditions with $FeCl_3$ or oxidation with $AgNO_3$ or HNO_3 led to the orthoquinone and the hydroxy-*p*-quinone due to loss of one or two methyl groups from the aromatic ring. These early studies pointed out the unusual reactivity of the 5-methyl group of α-tocopherol. Oxidation of α-tocopherol with benzoyl peroxide led to substitution of a benzoate on the 5-methyl group. A similar reaction occurs when diasobisisobutyronitrile is used as the oxidizing agent. The oxidation of α-tocopherol by tetrachloro-*o*-quinone in aqueous acetonitrile resulted in the formation of 9-hydroxy-α-tocopherone. When $FeCl_3$ was used as the oxidizing agent in the presence of α,α'-bipyridyl in ethanol, 9-ethoxy-α-tocopherone was formed. α-Tocopherolquinone can be reduced with Zn-HOAc or by catalytic hydrogenation to the hydroquinone or reductively cyclized to α-tocopherol with Zn-HBr. Reaction of α-tocopherolquinone with acetyl chloride resulted in the 5-chloromethyl-6-acetoxy derivative which has been converted to a variety of 5-methylsubstituted derivatives. Reaction of α-tocopherol with Br_2 led to the 5-bromomethyl derivative. When α-tocopherolquinone was treated with hydrochloric, phosphoric, citric or tartaric acid, in the absence of oxygen, a disproportionation took place forming α-tocopherol, α-tocored and other oxidation products. An interesting isomerization of α-tocored, the orthoquinone, occurs in the presence of aqueous HCl to yield the yellow *p*-quinone with the chroman ring closed. The 5-benzoyloxymethyl derivative upon treatment with HCl generates *o*-quinone methide which can be trapped by reaction with tetracyanoethylene or dihydropyran. Treatment of the 5-benzoyloxmethyl derivative with HCl in ethanol followed by sublimation yielded the 5-aldehyde of α-tocopherol. Recently, a series of phosphate derivatives of α-tocopherol or its model, 2,2,5,7,8-pentamethyl-6-chromanol, were synthesized. Tris(6-acetoxy-5-methyleneoxy-7,8-dimethyltocol)phosphate, tris(2,2,5,7,8-pentamethyl-6-chromanol)phosphate, 5-hydroxymethyl-2,2,7,8-tetramethyl-6-chromanol phosphate and the cyclic 5-methylenoxy-2,2,7,8-tetramethyl-6-chromanol phosphate, were prepared. These phosphates are of interest in view of a possible role of α-tocopherol in oxidative phosphorylation.

INTRODUCTION

Investigation into the chemistry of vitamin E started in 1927 with Evans and Burr (1) when they found that its biological activity was destroyed by bromination and not by hydrogenation. Evans et al. (2) later isolated α-, β- and γ-tocopherols from wheat germ oil in the form of solid allophonates. The chemical identification of α-tocopherol resulted from studies on its decomposition and oxidation products by Fernholz (3,4). Synthesis of the biologically active principle was performed by Karrer et al. (5) from trimethylhydroquinone and phytyl bromide. John, Smith et al. (6,7) confirmed the chroman nucleus of vitamin E. In due time, the structures of β and γ were ascertained and they were synthesized. Other tocopherols (5,7-dimethyltocol; 8-methyltocol; 7-methyltocol, 5-methyltocol and tocol) have been isolated from natural sources or synthesized since then. These all possess the saturated phytyl side chain. In addition, several tocopherols with unsaturation in the side chain have been isolated and synthesized.

[1]One of six papers to be published from the Symposium "Chemistry and Biochemistry of Tocopherols" presented at the ISF-AOCS World Congress, Chicago, September 1970.

OXIDATION REACTIONS OF THE TOCOPHEROLS

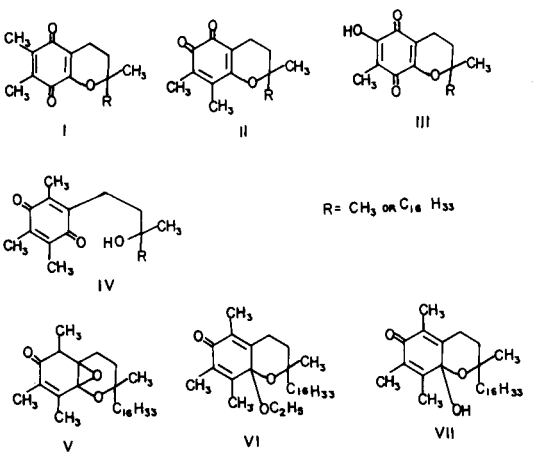
The most common reaction which the tocopherols undergo is one of oxidation. Interest in studying the oxidation reactions of the tocopherols are related to their use as anitoxidants and to interest in their biological role. Dimeric and trimeric oxidation products are excluded from this review.

In 1939, John and Emte (8) found that oxidation of α- or β-tocopherol with nitric acid in alcoholic solution gave an intense red coloration to the solution. Also silver nitrate oxidation in boiling alcohol yielded this red product. This reaction formed the basis of the Furter-Meyer (9) colorimetric method for analysis of tocopherols. Emmerie and Engel (10) have developed a colorimetric method for tocopherols based on oxidation with ferric chloride. John used the model compound 2,2,5,7,8-pentamethyl-6-chromanol, to study the oxidation reaction with silver nitrate. The red product obtained from the model was named chromanred 109 and that from α-tocopherol, α-tocored. Structure I was proposed for the red oxidation product by John.

This structure was later shown to be in error by Smith et al. (11) who showed that the red oxidation product, α-tocored, possessed Structure II. Compounds of Structure I could be formed from the red, orthoquinones, by treatment with hydrochloric acid. This latter isomerization was first noted by John and Emte (12).

In addition to this isomeric *p*-quinone, John and Emte (12) also isolated a hydroxy-*p*-quinone upon oxidation of α-tocopherol with silver nitrate in boiling ethanol. Structure III was assigned by John on the basis of elementary analysis of the product and its indicator properties (purple in base and yellow in acid). It was shown by Frampton et al. (13) that this hydroxy quinone (tocopurple) could also be produced by ferric chloride oxidation or by treatment of tocored with hydrochloric acid. These workers also showed that tocored could be produced by oxidation of tocopherol with ferric chloride in methanol at 50 C. Structure III for tocopurple was proved unequivocally in 1960 by Frampton et al. (14).

These oxidations under relatively mild conditions resulting in elimination of aromatic methyl groups are interesting and unusual in the field of organic chemistry. The extreme reactivity of the 5-methyl group of α-tocopherol and its model compounds becomes obvious as one studies their various reactions.

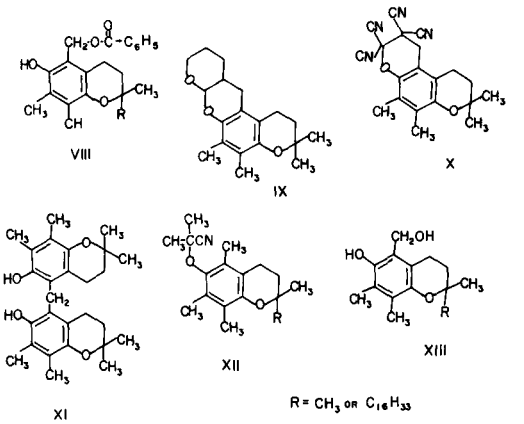


Mild oxidation of α-tocopherol with ferric chloride or silver nitrate yields α-tocopherolquinone (IV) (15). Boyer (16) isolated a product formed by oxidation with ferric chloride which was converted by acid to α-tocopherolquinone. Structure V was postulated for this product called α-tocopheroxide. Martius and Eilingsfeld (17) showed this structure to be in error with the correct structure as VI. More recently, Durckheimer and Cohn (18) studied the oxidation of α-tocopherol with tetrachloro-*o*-quinone or N-bromosuccinimide and prepared 9-hydroxy-α-tocopherone (VII). In the presence of alcohols, compounds of type VI were obtained. Deviation from a pH of 5.4 in either direction resulted in decomposition of VII to form α-tocopherol quinone.

Free radical initiated oxidation of α-tocopherol and its model compounds has led to interesting information about the mechanism of these oxidations and to new products. Inglett and Mattill (19) studied the oxidation of α- and γ-tocopherol and 2,2,5,7,8-pentamethyl-6-chromanol with benzoyl peroxide at 30 C. A surprisingly rapid oxidation of α-tocopherol occurs at this temperature yielding α-tocopherolquinone and a compound identified as VIII. γ-Tocopherol yielded the red, *ortho*-quinone. Goodhue and Risley (20) studied the reaction of *d*-α-tocopherol in hydrocarbon solvents with benzoyl peroxide. They obtained compound VIII which upon treatment with aqueous KOH yielded spirodienone dimer. These same workers (21) found that benzoyl peroxide oxidation of *d*-α-tocopherol in the presence of alcohols led to formation of 8a-alkoxy-α-tocopherones. Skinner and Parkhurst (22) confirmed the structure of the benzoyl peroxide oxidation product as VIII. Decomposition of the 5-benzoate with either hydrochloric acid in benzene or KOH in ethanol

yielded dimer and trimer. The ease of conversion of VIII to the intermediate quinone methide was used to prepare various tricyclic derivatives from dienophiles (IX and X). Treatment of the benzoate (VIII) with hydrochloric acid in ethanol followed by sublimation yielded the 5-aldehyde. Reduction of VIII with zinc-acetic acid gave the starting chroman. Refluxing VIII in aqueous hydrochloric acid in ethanol for several days under nitrogen gave a new product identified as dimer (XI).

Oxidation of 2,2,5,7,8-pentamethyl-6-chromanol with azobisisobutyronitrile, another free radical initiator, yielded a dihydroxy dimeric product (23) and a coupling product from the initiator and the phenoxy radical (24) (XII).



REDUCTION PRODUCTS

Treatment of α-tocopherolquinone with zinc and acetic acid causes reduction to the hydroquinone, which is very sensitive to air oxidation, and some cyclization to α-tocopherol (15). This reductive cyclization can also be obtained using zinc-hydrochloric acid. Catalytic hydrogenation of α-tocopherolquinone yields the hydroquinone.

HALOGENATION REACTIONS

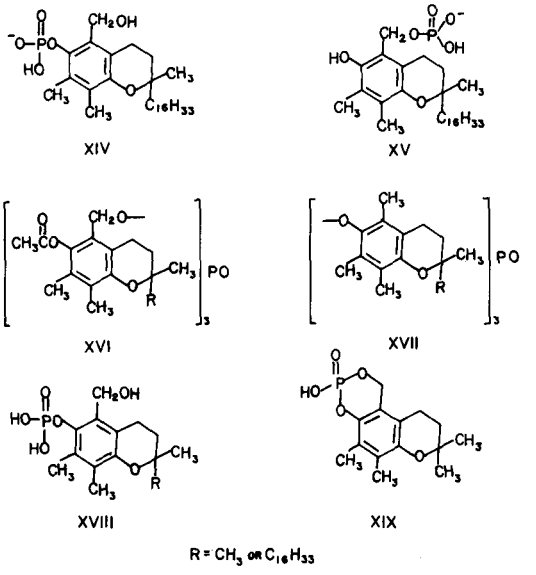
Bromination (21) of α-tocopherol led to the 5-bromomethyl derivative which was converted to the spirodienone dimer when treated with 1 N KOH. Treatment of α-tocopherol with acetyl chloride in benzene yielded the 5-chloromethyl-6-acetoxy derivative which has been converted to a variety of 5-methyl substituted derivatives of α-tocopherol (25).

ALKYLATION OF TOCOPHEROLS

Interest in conversion of γ-tocopherol to α-tocopherol has led to the study of alkylation of γ-tocopherol. Weisler (26) has hydroxymethylated γ-tocopherol using formalin and converted the crude 5-hydroxymethyl derivative to α-tocopherol by zinc-hydrochloric acid reduction. Haloalkylation of γ-tocopherol was described by Weisler and Chechak (27). Formylation of γ-tocopherol is covered by Weisler in other patents (28). Aminoalkylation (29) is covered by another patent (29). Recently, we prepared 2,2,7,8-tetramethyl-5-hydroxymethyl-6-chromanol (XIII) in analytically pure form as well as the corresponding α-tocopherol derivative.

ACID CATALYZED DISPROPORTIONATION REACTIONS

An interesting reaction of α-tocopherolquinone was discovered by Issidorides (30). She found that a disproportionation occurred when the quinone was treated with certain acids (phosphoric, citric or tartaric). Both α-tocopherol and α-tocored were formed from α-tocopherolquinone in this disproportionation which occurred in the absence of oxygen. These results again point to the unusual reactivity of the 5-methyl group of α-tocopherol.



PHOSPHATE DERIVATIVES

Phosphate derivatives of hydroquinones and chromans have been proposed as important intermediates in biological oxidative phosphorylation processes (31). Intermediates such as XIV and XV were proposed by Vilkas and Lederer (32).

In our laboratory we have synthesized a number of phosphate derivatives of α-tocopherol and its model; 2,2,5,7,8-pentamethyl-6-

chromanol. These are compounds XVI-XIX. The latter two compounds were synthesized from the 5-benzoyloxymethyl-6-hydroxychroman by treatment with phosphorus oxychloride followed by hydrolysis and by cyclization with dicyclohexylcarbodiimide.

EXPERIMENTAL PROCEDURES

2,2,7,8-Tetramethyl-5-hydroxymethyl-6-chromanol

2,2,7,8-Tetramethyl-6-chromanol (164.0 mg) was placed in a glass vial with 1 ml of water and 0.2 ml of 37% formalin. Nitrogen gas was bubbled through the mixture for 15 min and 25 mg of calcium hydroxide was added while the bubbling was continued and the glass vial was sealed with a flame. The vial was placed in a shaker and left over the weekend. The vial which now contained a thick slurry was acidified with acetic acid, extracted with ether and the ether washed with sodium bicarbonate solution and dried with sodium sulfate. Chromatography of the crude product on a thick layer silica gel plate with chloroform gave a band near the origin which was removed and eluted with ether. The product crystallized from ether-light petroleum ether mixture to give 60 mg of a white solid; mp 110-112 C.

Analysis calculated for $C_{14}H_{20}O_3$: C, 71.16; H, 8.53; Found: C, 71.35; H, 8.60.

5-Hydroxymethyl-γ-tocopherol

The procedure reported for the model compound was repeated exactly on 333 mg of γ-tocopherol. A colorless gum (52 mg) was recovered from chromatography.

Analysis calculated for $C_{29}H_{50}O_3$: C, 78.15; H, 11.08; Found: C, 78.25; H, 11.34.

2,2,7,8-Tetramethyl-5-bromomethyl-6-chromanol

Ten grams of model chromanol was dissolved in light petroleum ether and 9.2 g of bromine was added in light petroleum ether. After standing at room temperature for 0.5 hr, the solvent was removed in vacuo and the solid material remaining was recrystallized from light petroleum ether which gave a nearly quantitative yield of cream colored needles, mp 73-75 C.

Analysis calculated for $C_{14}H_{19}O_2Br$: C, 56.20; H, 6.40; Br, 26.71; Found: C, 56.16; H, 6.45; Br, 26.62.

Sodium methoxide converts this compound to the 5-methoxymethyl derivative (33).

Analysis calculated for $C_{15}H_{22}O_3$: C, 71.96; H, 8.86. Found: C, 71.77; H, 8.91.

Tris(2,2,5,7,8-pentamethyl-6-chromanol)phosphate

2,2,5,7,8-Pentamethyl-6-chromanol (2.2 g) was dissolved in hexane and 0.24 g of sodium hydride was added while stirring under nitrogen. After 1/2 hr a slight excess of $POCl_3$ was slowly added to the stirring slurry. The sodium chloride was filtered and the filtrate evaporated to a small volume, washed with sodium bicarbonate, dried with anhydrous sodium carbonate and ether added. Evaporation of the ether gave a white precipitate which was recrystallized from hexane-ether mixture to give 2 g of a white solid, mp, 174-175 C.

Analysis calculated for $C_{42}H_{57}O_7P$: C, 71.57; H, 8.15; Found: C, 71.57; H, 7.94.

Tris(6-acetoxy-5-methylenoxy-7,8-dimethyltocol)phosphate

5-Chloromethyl-γ-tocopherol acetate (6.08 g) and 1.67 g of yellow, silver phosphate were stirred in refluxing diglyme for 6 hr under a nitrogen atmosphere. During this time the yellow color of the silver phosphate changed to a grey-black. The solution was decanted into ice water and extracted with ether. The ether was removed and the brown product chromatographed on a silica gel column using light petroleum ether-ether mixtures. A center cut was rechromatographed on florisil and finally on Silica gel GF-254 (according to E. Stahl) thick layer plates with chloroform, giving a very viscous and almost colorless oil (in about 10% yield) which showed only one spot on thin layer chromatography; Silica gel GF-254 (according to Stahl)-$CHCl_3$, R_f = 10-23. $\gamma^{(\mu)}_{max}$ = 5.65 (CO) 6.30, 7.50, 7.95, 8.34, 8.96, 9.20, 10.15, 13.55 and 14.45.

Analysis calculated for $C_{93}H_{153}O_{13}P$: C, 73.97; H, 10.21; Found: C, 74.14; H, 10.00.

5-Hydroxymethyl-2,2,7,8-tetramethyl-6-chromanol phosphate

2,2,7,8-Tetramethyl-5-benzoyloxy-6-chromanol (2 g) was dissolved in dry pyridine (20 ml) and 2.4 ml of $POCl_3$ in 20 ml of dry pyridine was added to the ice cold, stirring mixture. The ice bath was removed and the mixture was allowed to stir under N_2 for 36 hr. The solution was poured into water, acidified and extracted with ether. The ether was washed with 6 N HCl, water and dried over sodium sulfate. Upon evaporation in vacuo a gum was isolated. This gum was dissolved in alcohol and NaOH (aqueous) added. The precipitate was filtered and dissolved in water and HCl added, extracted with ether and evaporated in vacuo. The white solid obtained was recrystallized from ethyl acetate-ether-petroleum ether to yield a solid; mp 145-151 C. NMR in pyridine and D_2O was consistent with the above structure as was the IR spectrum. The yield was 705 mg (38%, theory). A titration of this material

gave an equivalent weight of 164 or a molecular weight of 328 (calculated, 316.3).

The analysis was calculated for $C_{14}H_{21}O_6P$: C, 53.16; H, 6.69; P, 10.03; Found: C, 53.25; H, 6.60; P, 9.70.

Cyclic 5-methylenoxy-2,2,7,8-tetramethyl-6-chromanol phosphate

The 5-hydroxymethyl-2,2,7,8-tetramethyl-6-chromanol phosphate (250 mg) was added to 5 ml of pyridine and stirred until dissolved. One milliliter of water and 1 g of dicyclohexylcarbodiimide was added and the mixture stirred over the weekend. Water was added and the dicyclohexylurea filtered off. The filtrate was extracted with ether three times and then made acidic with HCl. Washing with water, drying with sodium sulfate and evaporation in vacuo gave a glassy gum which crystallized on treatment with light petroleum ether after refrigerating for one week. Recrystallization from ethanol-ethyl acetate gave 78.6 mg of a white solid; mp 230-233 C (dec.).

The analysis was calculated for $C_{14}H_{19}O_5P$; C, 56.45; H, 6.44; P, 10.38; Found: C, 55.90; H, 6.29; P, 10.22.

The molecular weight (in EtOH) by osmometer was 284 (calculated, 298).

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Modern Procedures for the Analysis of Tocopherols[1]

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ABSTRACT

The development of reliable assay methodology for the tocopherols has been an evolutionary process which has required over 40 years to reach its present state. Today the analyst has at his disposal a choice of reliable and accurate methods of analysis for the eight tocopherols now known to exist in nature. The general sequence of procedure and the precautions to be observed are described. After suitable extraction procedures and careful saponification techniques, the tocopherols in the non-saponifiable fraction can be assayed by a variety of chromatographic procedures including paper, thin layer (TLC), column and gas liquid chromatography (GLC). The current methodology of these procedures is reviewed in some detail with special emphasis on the more frequently used TLC and GLC techniques. The earlier methods of GLC assay separated only the mono-, di- and trimethylated tocopherols but methods are now available which provide separation of all the tocopherols. These developments were made possible by better columns and the use of derivatives of tocopherols. In addition to good separation, the GLC method is the most sensitive available for the quantitation of the tocopherols. Spectrophotometric measurements based on Emmerie Engel type reactions have been most frequently used for the final quantitative analysis of the tocopherols separated by paper, TLC, or column chromatography.

INTRODUCTION

Publications in the area of vitamin E research which contain information on new or modified tocopherol assay procedures still appear at a frequent rate, even though vitamin E has been with us for over 40 years. The development of reliable assay methodology has been an evolutionary process which has achieved its greatest rate of development in the last 10 years. If the analyst's interest is primarily α-tocopherol, there are a variety of reliable and accurate methods at his disposal. The assay of natural products for all eight known tocopherols, however, is a much more complex affair and, therefore, will probably continue to receive attention by analytical biochemists.

A tremendous number of vitamin E publications have appeared over the past 40 years, many of which contain assay procedures for the tocopherols. To the research worker who may be concerning himself for the first time with tocopherol assay procedures, entrance into the literature of this field can be a bewildering experience. Even those individuals who have been involved in the field of vitamin E find the task of compiling a review of tocopherol assay procedures a difficult one. The most recent review in this field was a short one prepared by Bunnell in 1969 (1). A more extensive review was published by the same author in 1967 (2). Earlier reviews (3-5) are also available to aid the analyst. This review will attempt to emphasize the more recent developments in tocopherol analytical procedures and hope, thereby, to serve as a supplement to previous efforts.

For success in tocopherol assays, certain precautions are essential. Protection of tocopherols from oxidation during the various steps of an analytical procedure is of paramount importance and our vigilance in this respect can never be relaxed. Conditions which catalyze the rate of oxidation of tocopherol are light, heat, alkaline pH and metals. A maximum effort should therefore be made to exclude oxygen when a procedure in the course of the assay involves any of the aforementioned conditions, or conversely, when oxygen is present, to avoid any of these conditions. Additionally, exposure of small amounts of tocopherol in thin films to air should be minimized. Since most of our methods for the quantitative estimation of tocopherol depend on its reducing property, a property certainly not unique to tocopherols, a second serious source of error is the presence of reducing artifacts. This source of error has probably been responsible for more inaccuracies in tocopherol assays than any other. Avoidance of this error requires careful attention to detail. Besides the obvious requirement of cleanliness of glassware, all sol-

[1]One of six papers being published from the "Chemistry and Biochemistry of Tocopherols," presented at the ISF-AOCS World Congress, Chicago, September 1970.

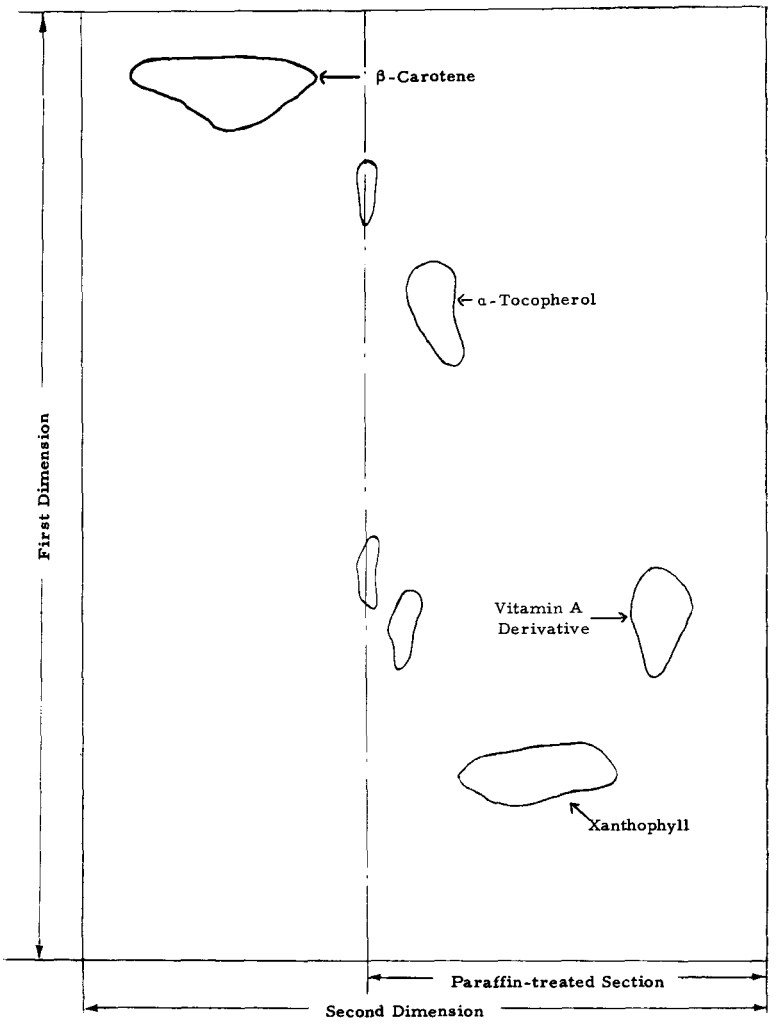

FIG. 1. Two-dimensional paper chromatogram of animal serum (13).

vents should be carefully purified or checked for purity with regard to the presence of oxidizing or reducing substances. Contact of samples with other substances should be avoided. Examples of these are rubber, which can introduce foreign reducing substances, and silicone stopcock grease, which can adversely effect chromatographic procedures. With regard to contact with rubber, the experience reported by Morton (6) should make humble analysts of us all. In an investigation of the lipid components of human bone marrow, γ-tocotrienol was reported as present in this tissue. On reinvestigation, however, it was discovered that the γ-tocotrienol came from the rubber membrane used in the dialysis of the bond marrow lipids.

The analytical steps usually employed in tocopherol assay can be roughly divided into the sequence: extraction; separation of fats, e.g., saponification; chromatography—paper, thin layer, column, gas liquid; measurement—colorimetric, e.g., Emmerie Engel, GLC, spectrophotometric, spectrofluorometric. Since the methods for the extraction of samples and the removal of lipids by saponification or molecular distillation have been well covered in previous reviews, primary attention will be given to methods of chromatography and measurement of the tocopherols.

CHROMATOGRAPHIC PROCEDURES

Paper

Although paper chromatography is gradually being replaced by thin layer (TLC) and gas liquid chromatography (GLC), this technique

played a very important historical role in the separation and identification of new tocopherols. The first efforts in this area (7,8) employed one-dimensional chromatography on papers impregnated with liquid paraffin. Based on the later refinements of Green et al. (9), a standard method of vitamin E assay was proposed by a vitamin E panel of the Analytical Methods Committee in England (10). This method employed adsorption chromatography in the first dimension on zinc carbonate impregnated paper with a benzene-cyclohexane developing solvent and reversed phase partition chromatography in the second dimension using ethanol-water on paraffin impregnated papers. This method was used successfully by many investigators with some modification for special cases.

Some recent applications of paper chromatography include the work of Jensen (11) who determined the tocopherol content of seaweed and seaweed meal and Bayfield et al. who measured the tocopherol content of pastures, feed and feces (12) and blood serum (13) without prior saponification. Jensen used circular chromatography on S & S 18 cm papers impregnated with 20% alumina. The papers were used directly without activation or were dipped in 0.02% fluorescein. The chromatograms were run in Petri dishes with diethyl ether (20-25%) in petroleum ether as the developing solvent. After location, the tocopherol-containing rings were cut out, eluted and assayed. Prior saponification of the lipid extract did not appear necessary. Bayfield et al. assayed both β-carotene and α-tocopherol in biological materials using a two-dimensional system. The 28 cm square zinc carbonate impregnated papers (10) were developed in light petroleum (40-60°) - acetone 99:1 (for serum) in the first dimension. The spots were then located quickly with UV light and the unused part of the paper dipped in 3% paraffin in light petroleum. Development in the second dimension was then carried out using 90% aqueous methanol. Time required was 75-90 min for the first dimension and 2 1/2 hr for the second dimension. A typical chromatogram which they obtained for sheep serum is shown in Figure 1. They were able to quantitatively estimate β-carotene in addition to α-tocopherol as well as to qualitatively show the presence of other carotenoids and probably vitamin A. These investigators considered the optimum amount of both α-tocopherol and β-carotene to be 10 μg for a reliable quantitative assay. Recoveries of α-tocopherol and β-carotene were about 90%. In the case of pasture feed and feces, they were also able to easily detect the presence of α-tocopheryl hydroquinone which had a high R_f value in the second dimension. In an earlier publication, Herting and Drury (14) used two-dimensional paper chromatography according to the procedure of the Analytical Methods Committee (10) for the assay of human blood serum or plasma. They also claimed that prior saponification of the plasma was not necessary. Recovery of α-tocopherol was 99% after correcting for a 13% loss of α-tocopherol alone on the paper. Further aspects of the assay of tocopherol in blood plasma will be discussed in the section on TLC, a method which is rapidly displacing paper chromatography due to greater simplicity and speed.

TABLE I

Developing Solvents for Silica Gel G TLC of Tocopherols

Solvent	References
One-dimensional	
Benzene	15-18
Benzene 80:heptane 20	19
Chloroform	16,20
Chloroform 1:cyclohexane 2	16
Hexane 92.5:ethyl acetate 7.5	21
Petroleum ether 80:ethyl ether 20	22
Petroleum ether 85:isopropyl ether 12: acetone 4:ethyl ether 1:acetic acid 1	23
Two-dimensional	
Chloroform Hexane 80:isopropyl ether 20	24
Chloroform Petroleum ether 80:isopropyl ether 20	25
Cyclohexane 80:ethyl ether 20 Benzene 99:methanol 1	26

Thin Layer

The use of TLC for the analysis of tocopherols has made rapid advances in the last 10 years so that it is now one of the most important analytical tools in this field. Silica gel G has enjoyed the widest usage but alumina, and to a lesser extent, magnesium phosphate have also been used as coatings for thin layer plates in tocopherol analysis.

Silica gel G plates are prepared in the usual manner with a coating thickness of about 250 μ and dried at about 100 C for 1/2 to 1 hr. Fluorescein is often incorporated during the preparation of the plates to enable the UV visualization of the tocopherol spots. A wide variety of developing solvents have been used with Silica gel G in both one- and two-dimensional chromatographic systems. A list of solvents which have been used for TLC of tocopherols on Silica gel G plates is shown in Table I. The choice of a mobile phase is usually dic-

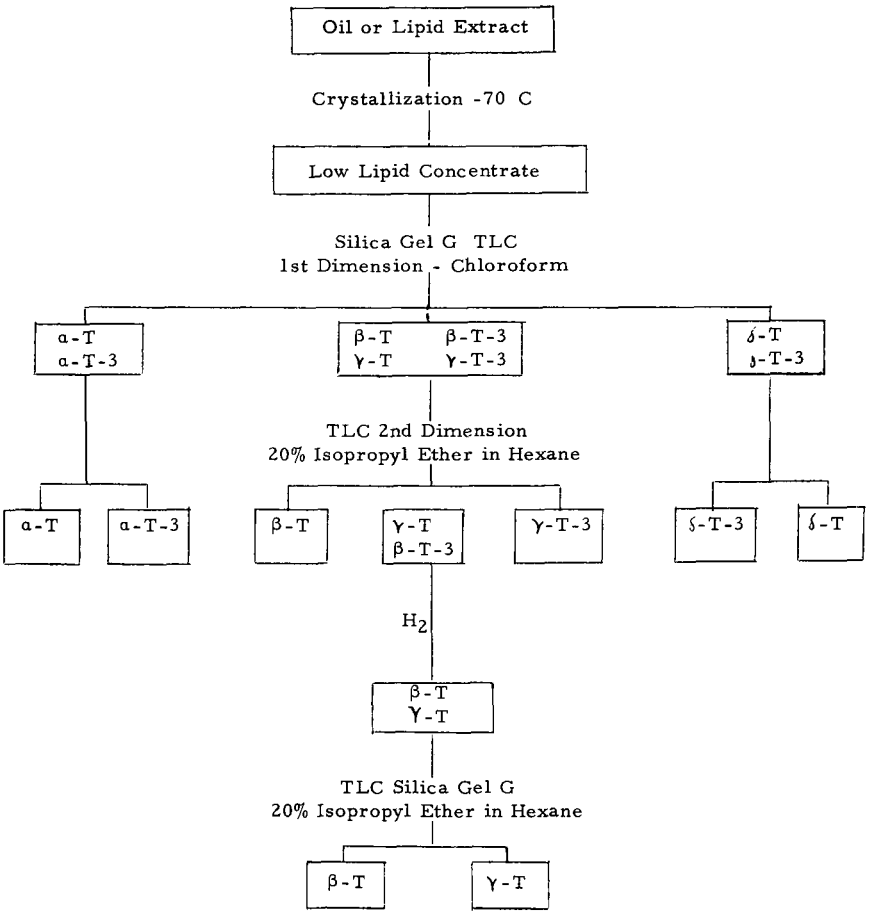

FIG. 2. Tocopherol assay procedure of Chow et al. (24).

tated by the pattern of tocopherols present, the impurities which must be separated, and whether the investigator wishes a quantitative estimate of all the individual tocopherols which are present in the sample. In handling an unknown sample, it is usually advantageous to run a qualitative two-dimensional TLC with tocopherol standards to learn the pattern of the tocopherols and interfering substances which may be present (25). An attempt was made to tabulate R_f values for the various tocopherols in different solvent systems but these often do not agree too well among investigators. Tabulations of R_f values, however, can be found in several references (20,27).

Seher (28) first demonstrated that the tocopherols could be separated by TLC but his system did not separate β- and γ-tocopherols. Good separation of α-, β-, γ-, and δ-tocopherols was later achieved by Stowe (23) using a five component solvent system. Rao et al. (29) successfully used Stowe's technique to assay the tocopherols in a variety of vegetable oils. Pennock et al. (30) and Whittle and Pennck (25) introduced further refinements of Silica gel G TLC of the tocopherols by using a two-dimensional system to separate the tocopherols and tocotrienols. The mono-, di- and trimethylated tocopherols were separated using chloroform in the first dimension. Chromatography in the second dimension using 20% isopropyl ether in light petroleum ether separated α-tocopherol from α-tocotrienol, β-tocopherol from β-tocotrienol plus γ-tocopherol and from γ-tocotrienol, and δ-tocopherol from δ-tocotrienol. β-Tocotrienol and γ-tocopherol overlapped each other but when these two tocopherols occurred together they could be separated by reversed

phase chromatography or paraffin impregnated paper. The two-dimensional TLC system, refined by Whittle and Pennock (25), has proven to be an excellent method for established the presence of tocotrienols.

Chow et al. (24) developed a method for the assay of free and esterified tocopherols and tocotrienols and applied it to a variety of cereals and rubber latex. Their method made use of the Pennock et al. (30) system of two-dimensional TLC. An important feature of their method was the removal of the bulk of the lipids by low temperature (-70 C) crystallization rather than saponification. During the course of the development of their methods, it was found that saponificiation destroyed a significant amount of tocotrienols. The use of low temperature fat crystallization circumvented this difficulty. These investigators also solved the problem of assaying β-tocotrienol and γ-tocopherol when they occur together. The combined β-tocotrienol-γ-tocopherol spot was removed from the plates and hydrogenated by a micro procedure. The resulting mixture of β- and γ-tocopherols was then separated by rechromatography in the isopropyl ether-hexane system. Thus a method for determining all eight tocopherols was developed. The tocotrienol esters, which occur in rubber latex, were reduced by $LiAlH_4$ to the free form with minimum loss. Their method has much to recommend it for the complete analysis of tocopherols in natural products. The overall scheme for the analysis of a lipid extract is shown in Figure 2.

TLC on Silica gel G has been used for the assay of tocopherol in a wide variety of materials such as vegetable oils (20,24,25,29), leaves (18,19), alfalfa (21), blood serum (15,31,32), liver (17), and tocopherol oxidation products (16). In the case of blood serum, Horwitt et al. (32) reported that the losses of tocopherol were excessive when blood serum containing less than 0.6 mg/100 ml was assayed by TLC. He suggested that some other acceptable method for total tocopherol determination should also be run as a check on the TLC assay.

Alumina has also been used for the TLC of tocopherols. Herting and Drury (33) described the use of inactivated alumina Chromatogram sheets for the tocopherol analysis of a wide variety of biological samples using one-dimensional chromatography with a solvent system of benzene-diethyl ether (50:50). Recovery of tocopherol standards on these sheets was reported as 90-95% and the analytical results were comparable to column chromatography on magnesium phosphate. In the case of blood serum, chromatography of unsaponified serum was not as good on alumina sheets as it was on Silica gel G sheets, but with saponified serum, alumina sheets were better. The same investigators (34) applied TLC on alumina sheets to the assay of tocopherol in a variety of cereals. Two-dimensional chromatography was used in this study with solvent system of benzene-diethyl ether (90:10) in the first dimension and light petroleum ether-isopropyl ether (80:20) in the second dimension. Recoveries in this two-dimensional system were in the 80-81% range.

Schmidt (35) also used TLC on neutral alumina plates for the analysis of tocopherols in oils and fats. Chloroform was used as the developing solvent in a one-dimensional system. A special feature of Schmidt's method was the inactivation of the alumina by application of methanol to the tocopherol spots, and then extraction of the alumina scraped from the plates for 1/2 hr under reflux with ether. The technique was apparently necessary for the complete extraction of the tocopherols, particularly γ- and δ-tocopherols.

Roughan (27) carried out a special study of TLC systems to improve the separation of α- and β-tocopherols. A variety of thin layers were tried, and the best systems were aluminum oxide-zinc carbonate (3:1) with chloroform as the solvent or Silica gel G-zinc carbonate (2:1) with benzene-cyclohexane (30:70).

Column

There have been few significant advances in the use of column chromatography for the analysis of tocopherols since the last review of tocopherol analytical methods (2). The excellent method of Bro-Rasmussen and Hjarde (36,37) has been handicapped by the difficulty of obtaining batches of secondary magnesium phosphate that will give good separation of the tocopherols. This has forced many investigators to the use of TLC even though recovery of tocopherol using this column chromatographic technique is superior to TLC. Dicks-Bushnell (38) encountered difficulty with *sec*-magnesium phosphate columns and, therefore, investigated the use of Florisil and silicic acid for the column chromatography of tocopherols. Although some separation of tocopherols was achieved with these adsorbents, neither was completely satisfactory. In a subsequent study by Dicks-Bushnell and Davis (39) use was made of column chromatography on Florisil followed by paper chromatography for the tocopherol analysis of infant formulas and cereals. These workers found that spotting of less than 10 μg of tocopherol gave poor results in quantitative assays using paper chromatography. This is in agreement with Horwitt et al. (32) who also

TABLE II

Relative Retention Data for Some Tocopherols, Their *p*-Quinones and Their Trimethylsilyl Ethers (50)

Compound	Parent compound	*p*-Quinone	Trimethylsilyl ethers
Tocol	1.00	1.00	0.80
5-Methyltocol	1.26	1.16	1.07
7-Methyltocol	1.11	1.27	0.91
8-Methyltocol	1.08	1.30	0.83
5,7,8-Trimethyltocol	1.69	1.86	1.66

reported difficulties in recoveries with small amounts of tocopherol on TLC.

Ackman and Cormier (40) used alumina-zinc carbonate columns, according to the method of Bieri et al. (41), for the analysis of α-tocopherol in Atlantic fish and shellfish. A special feature of their method was a rather unique column arrangement which maintained a nitrogen atmosphere, permitted application of a partial vacuum to speed up column flow and facilitate removal of the solvent in the collected fractions without removal of the collecting tubes from their racks. This apparatus should find application in the column chromatography of any oxygen labile substances.

Ikeda and Taguchi (42) employed column chromatography on acid alumina in their assay procedure for tocopherol in fish tissues. They demonstrated good separation of α-tocopherol from ubiquinone. Nazir and Magar (43) have also used alumina columns for the assay of tocopherol and ubiquinone in shark tissues.

Column chromatographic techniques will probably continue to find application in the analysis of tocopherols, either as the principle technique or coupled with other methods of chromatography such as paper or TLC.

Gas Liquid

The use of GLC for the analysis of tocopherols has increased considerably since it was first reported about 10 years ago. It offers the advantage of separation and quantitation of the tocopherols in one step. It also offers greater sensitivity than previous spectrophotometric methods which were limited to levels of about 1 μg. Improvements in methodology are continually being made so that its dominant role in tocopherol analysis in the future is assured. For a summary of investigations in the field up to about 1965, the reader is again referred to a previous review (2).

Tocopherol analysis by GLC was quickly accepted for pharmaceutical products (44,45) and this method is under collaborative study and will undoubtedly be the official method in the near future. It has the advantage of ease and sensitivity and can easily determine both tocopherol and the acetate esters in the same mixture.

Ishikawa and Katsui (46) reported on a method for the assay of tocopherols which involved acetylation to the acetate esters and chromatography on 1.5% SE-30 on chromosorb W, hydrogen flame ionization detection and nitrogen carrier gas. Squalene was used as an internal standard. Separation of β- and γ-tocopherols was not achieved. These authors also reported relative retention times of the trimethylsilyl ethers of the tocopherols. This method was used for the assay of tocopherols in soybean oil (47). Eisner et al. (48) employed GLC on 1.5% SE-30 on silanized Gas Chrom P in the analysis of aliphatic alcohols, tocopherols

TABLE III

Relative Retention Data for Substituted Tocols and Their Esters (50)

Compound	Parent compound	Acetate	Trifluoro-acetate	Propionate	Pentafluoro-propionate
Tocol	1.00	1.13	0.46	1.50	0.44
5-Methyltocol	1.26	1.46	0.62	1.87	0.62
7-Methyltocol	1.11	1.26	0.54	1.68	0.53
8-Methyltocol	1.08	1.26	0.52	1.65	0.50
5,7-Dimethyltocol	1.36	1.63	0.70	2.14	0.70
5,8-Dimethyltocol	1.35	1.60	0.67	2.10	0.66
7,8-Dimethyltocol	1.39	1.64	0.68	2.14	0.67
5,7,8-Trimethyltocol	1.69	2.02	0.88	2.65	0.88

and triterpenoid alcohols in butter and vegetable oils. Column chromatography on Florisil was used prior to GLC. Nair and coworkers have made significant advances in improving the sensitivity and reproducibility of GLC techniques for tocopherols (49,50). Their work involved an extensive investigation of the GLC properties of the tocopherols, tocopherol quinones, the trimethylsilylethers, and the acetate, propionate, trifluoracetate and pentafluoropropionate esters. The principle column support used was a mixture of SE-52 and XE-60 on Gas Chrom P. Relative retention times of the quinones and trimethylsilylethers of some tocopherols are shown in Table II and for a variety of esters in Table III. No data were given for the tocotrienols. These investigators found *bis* (trimethylsilyl) acetamide a preferred reagent for preparing trimethylsilylethers. Nair and Luna (51) combined these GLC techniques with mass spectrometry and IR spectroscopy for the identification of α-tocopherol in tissues. Trifluoroacetate and trimethylsilyl derivatives of α-tocopherol, isolated from tissues, were proven to be identical to the same derivatives made from standard α-tocopherol. These GLC tocopherol analytical techniques were used in connection with a study of lipofuscin-like pigments in the rat adrenal gland (52).

Slover et al. (53) applied a GLC method for the identification and estimation of tocopherols and tocotrienols as their trimethylsilyl ethers. They used silanized glass columns packed with either 0.5% Apiezon L or 2% SE-30 on Anakrom. The methods were applied to preliminary studies of the tocopherols present in soybean oil, whole wheat flour, wheat germ oil and corn meal. Some tocotrienols were found in all samples except soybean oil.

An ingenious combination of radio label techniques, TLC and GLC were used by Bieri et al. (54) in solving the problem of the determination of α-tocopherol in erythrocytes. Preliminary experiments had indicated erratic results and only 30-60% recovery of ^{14}C-α-tocopherol added to erythrocytes. Further studies indicated that considerable oxidation of the tocopherol occurred during extraction. In view of this, the decision was made to oxidize the tocopherol and determine the resulting tocopheryl-quinone. Studies with the oxidation of α-tocopherol-^{3}H in the absence and presence of lipids from erythrocytes also indicated a loss of α-tocopheryl-quinone. This loss could have been due to the formation of an addition compound of tocopherylquinone and unsaturated fatty esters similar to the findings of Komoda and Horada (55) in working with tocopherol oxidation products in soybean oil. In order to correct for this loss of α-tocopherylquinone, a known amount of α-tocopherol-^{3}H was added to the washed cells during extraction. The assay solution was then counted in addition to the GLC analysis in order to make the correction for overall losses of α-tocopherol. The GLC analysis of α-tocopherylquinone was carried out using hydrogen flame detection with Glass U columns packed with 3% QF-1 on silanized Supelcoport. The response of the detector was linear in the range of 0.025-0.25 μg of α-tocopherylquinone. Submicrogram amounts of α-tocopherol could, therefore, be detected in 0.5 ml of packed erythrocytes. (A flow chart of the procedure is shown in Figure 3.) Bieri and Poukka (56) then applied this technique to a study of the red cell content of α-tocopherol in normal subjects and patients with abnormal lipid metabolism.

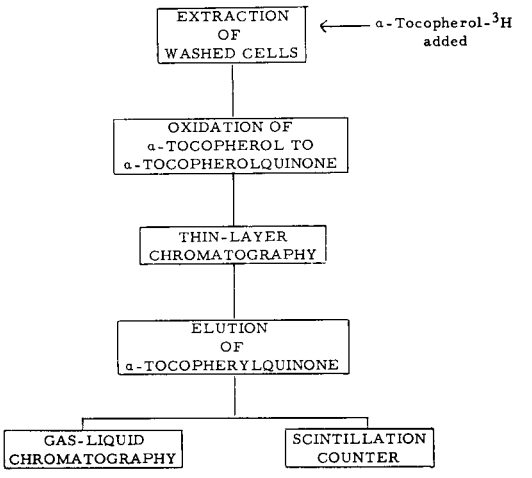

FIG. 3. Analysis of erythrocytes for α-tocopherol (54).

MEASUREMENT OF THE TOCOPHEROLS

The quantitative measurement of tocopherols by GLC has already been discussed and at the present time this method has the greatest sensitivity. Colorimetric or spectrophotometric measurement, however, has been the most widely used method. Reviews previously cited cover the details of these methods. The procedure used most widely at the present time is Tsen's (57) modification of the Emmerie Engel method using bathophenanthroline as the chromogenic reagent. This has increased the sensitivity of the original Emmerie Engel procedure by a factor of about 2.5. The stable free radical α,α'-diphenyl-β-picrylhydrazyl (DPPH) offers interesting possibility as a colorimetric reagnet in tocopherol analysis. This reagent was first

proposed by Blois (58) and further developed by Glavind (59) for the assay of antioxidants in animal tissue. Glavind and Holmer (26) later described a method for the TLC determination of antioxidants using DPPH. Since the deep violet color of the DPPH reagent is reduced by tocopherol, the color fades to yellow giving a decrease in optical density at 520 nm. For visualization of spots on TLC plates, the tocopherols or antioxidants show up as bright yellow spots on a violet background. A quantitative procedure using the DPPH reagent was also described by Glavind and Holmer. Recently Boguth and Repges (60) also described a spectrophotometric procedure for the determination of α-tocopherol with DPPH. The $E^{1\,cm}_{520}$ for DPPH is 1286 and the decrease in extinction with concentration obeys the Lambert-Beer law.

Direct measurement of tocopherols by UV spectrophotometry has found only limited use due to the low extinction values of the tocopherols and to the problem of interfering substances. Spectrofluorometric methods are better and were developed by Duggan (61) for the assay of tocopherol in blood serum. Duggan's method was further developed by Hansen and Warwick (62) as a fluorometric micro method for serum tocopherol.

Other procedures which have recently been proposed for the determination of α-tocopherol are a polarographic technique (63) and an oxidimetric procedure (64) which measures the content of ferrous ions, formed by oxidation of α-tocopherol with ferric chloride, by titration with dichlorophenolindophenol.

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The Quantitative Analysis of Sphingolipids by Determination of Long Chain Base as the Trinitrobenzene Sulfonic Acid Derivative[1]

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ABSTRACT

A spectrophotometric procedure has been developed for the determination of microquantities of sphingolipids. The assay system involved includes the methanolysis of the fatty acid moiety of the sphingolipid with boron trifluoride in methanol to yield a long chain base containing a free amino group. The long chain amine, sphingosine, is then extracted into an organic phase and reacted with aqueous trinitrobenzene sulfonic acid to yield a product with an absorption maximum at 340 mμ. Lipid peroxidation products and silicic acid do not seriously interfere in the 340 region. A wide variety of pure sphingolipids yielded equivalent optical densities per mole of sphingolipid. Sphingolipids assayed included cerebroside, cerebroside sulfate, ganglioside and sphingomyelin.

INTRODUCTION

Procedures for the quantitative analysis of sphingolipids have been based on the determination of the long chain base of sphingosine and the analysis of other sphingolipid components. A direct analysis for sphingosine is the preferred analytical approach to sphingolipid analysis since all known sphingolipids contain one molecule of sphingosine per molecule of sphingolipid. Moreover, the determination of mole ratios of associated chemical components with reference to sphingosine is essential for the characterization of various sphingolipid species. Several procedures have been employed for measuring sphingosine, including gas liquid chromatography of the aldehyde products of oxidized sphingosine (1), and the spectrophotometric determination of dye-sphingosine complexes (2,3). Alternative approaches have been to measure the concentration of components associated with specific sphingolipids; these include phosphorus (sphingomyelin) (4), neuraminic acid (gangliosides) (5), sulfate (sulfatides) (6), and galactose (cerebrosides) (7). All of these approaches feature some disadvantages. Hexose analyses by procedures using sulfuric acid are particularly difficult since the parent sphingolipid must be employed as an absolute standard for quantitation because of the many side reactions experienced with different components unique to a number of sphingolipid species (8). Other disadvantages of previous methods are numerous chances for errors during multiple extraction operations and solvent transfers.

The procedure described in this paper was developed from an earlier described method for lipids containing free amino groups using trinitrobenzene sulfonic acid (TNBS) (9). This new procedure, modified for sphingolipids, yields identical optical densities, on a molecular weight basis, for the sphingosine-TNBS reaction products with all sphingolipids assayed. This assay is rapid and sensitive to 5 μg or less of cerebroside. Absolute standards are not required and samples may be assayed directly in the presence of different chromatographic media. All reaction and extraction steps are carried out in one reaction tube.

MATERIALS AND METHODS

The following commercial sources of reagents were employed in this study: trinitrobenzene sulfonic acid, sodium dihydrate (Pierce Chemical Co., Rockford, Ill.). [Trinitrobenzene sulfonic acid (Eastman 8746, Distillation Products Industries, Rochester, N.Y.) must be purified by recrystallization from water after conversion to the sodium salt with sodium bicarbonate.] Hydroxide of Hyamine, 1 M in absolute methanol (Packard Instrument Co., Downer's Grove, Ill.); silica gel, plain (Warner-Chilcott Labs, Richmond, Calif.)

The 7% boron trifluoride (BF_3) solution was prepared by cooling a flask containing redistilled absolute methanol in a dry ice-acetone bath and adding BF_3 gas (The Matheson Co., East Rutherford, N.J.) through a Teflon tube to make 7% BF_3 w/w.

The sphingolipid samples, obtained from Supelco., Bellefonte, Penna., were monitored for purity by appropriate thin layer chromatography (TLC) systems. Lipid preparations with

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traces of impurities were excluded. Samples of pure disialogangliosides (HG-2 and HG-4) were generously supplied by Dr. McCluer (W.E. Fernald State School, Waverly, Mass.).

Mixtures of cerebroside, cerebroside sulfate and sphingomyelin were separated by one-dimensional (1D) TLC using silicic acid with 10% magnesium silicate or silica gel, plain with the solvent systems chloroform-methanol-water (65:25:4) or chloroform-acetone-methanol-acetic acid-water (3:4:1:1:0.5) (10). The individual sphingolipid classes were detected by lightly spraying the air-dried TLC plate with distilled water and outlining the hydrophobic areas. The labeled TLC plates were stored over magnesium perchlorate overnight and the appropriate areas were transferred quantitatively into a 16 x 100 mm Pyrex culture tube, Corning Glass Works, Corning, N.Y.

EXPERIMENTAL PROCEDURES

An aliquot of the lipid mixture was evaporated under nitrogen in a Pyrex culture tube. Alternatively, lipid-silicic acid samples from TLC plates were transferred to a culture tube. One ml of 7% BF_3 in methanol was added. The tubes were sealed with rubber-backed Teflon-lined plastic caps (GCMI 415-15, Corning Glass Works, Corning, N.Y.). The tubes were heated at 110 C for 1 hr, then cooled. The tubes were uncapped and 2 ml benzene was added, followed by 1 ml of the buffered Hyamine solution (1M $NaHCO_3$, 0.2 M Na_2CO_3, containing 2.5% 1M Hyamine hydroxide). The tubes were capped and extracted vigorously for 5 min on a mechanical shaking machine. Samples containing silicic acid (TLC samples) were centrifuged at 1000 g for 10 min to sediment the silicic acid after which the total liquid phase was poured into a clean Pyrex culture tube. Next, 0.5 ml of 0.1% aqueous TNBS was added, the samples were capped, mixed immediately with a vortex mixer and incubated 1 hr at room temperature in the dark. Finally, 1.0 ml 10% aqueous hydrochloric acid (1.2 N) was added to quench the unreacted TNBS color and the samples were reextracted for 5 min to separate the liquid phases. The clear supernatant solution is read at 340 mμ against a reagent blank. For best reproducibility mix in cuvette before final reading. With samples separated by TLC, a silicic acid blank is carried through all steps.

Highly colored samples may be color-corrected by omitting the TNBS reagent in a replicate. With such preparations a "blank" consists of an equivalent aliquot of the sample except that 0.5 ml of distilled water is substituted for the TNBS reagent. Such color correction blanks

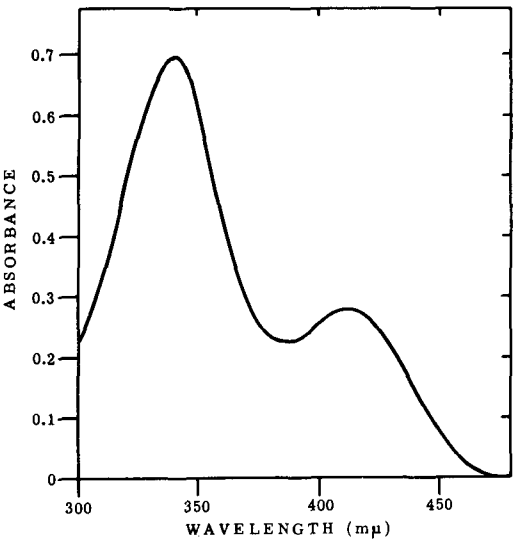

FIG. 1. Absorption spectrum of the TNBS derivative of 100 μg bovine brain cerebroside. Obtained as difference spectrum between bland and cerebroside sample.

are generally not necessary with glycolipid samples eluted from columns or separated from other polar lipids by TLC. The absolute specificity of this method for sphingolipids is maintained by a chromatographic separation procedure, although the presence of phosphatidylethanolamine (PE) or phosphatidylserine (PS) may be corrected by a previously published method (9) for amino-containing lipids. That procedure (9) is specific for lipids with free amino groups and completely excludes all sphingolipids (Bacteria and invertebrates may contain ceramide phosphorylethanolamine and some invertebrates, ceramide phosphoroethanolamine.) except free sphingosine since the transacylation step is not included. With the present procedure, traces of free amino-containing lipids may be determined by omitting the transacylation step, that is, altering the addition sequence of the reagents (adding 2 ml benzene to the sample, followed by the buffered Hyamine, then the methanolic 7% BF_3 mixture, and the TNBS reagent), and continuing with the procedure as described above. Under these conditions, only lipids with free amino groups are detected. This optical density value can be subtracted from the optical density obtained after sphingolipid analysis to correct for the true sphingolipid content.

RESULTS

The absorption spectrum was determined for 100 μg of cerebroside with a reagent blank as

TABLE I

Experimental Variation of the TNBS Procedure in Optical Density Units at 340 mμ[a]

	Test tube system				Thin layer system			
	Days	n	CV[b]	F ratio[c]	Days	n	CV[b]	F ratio[c]
Reagent blank	1	10	3.11	1.6030[b]	1	5	3.18	6.0230[f]
	2	5	1.92		2	5	3.09	
	3	5	2.37		3	5	3.49	
	4	5	2.03		4	5	3.49	
	5	5	2.60		5	5	2.95	
			2.40				3.24	
Cerebroside samples	1	10	1.32	2.1105	1	5	3.23	6.2684[f]
	2	5	2.63		2	5	1.53	
	3	5	0.48		3	5	0.69	
	4	5	1.91		4	5	1.44	
	5	5	1.45		5	5	1.57	
			1.55				1.69	
Optical density differences	1	10	2.98	0.8740[d]	1	5	4.88	2.128[d]
	2	5	3.97		2	5	4.33	
	3	5	1.98		3	5	3.23	
	4	5	4.41		4	5	0.83	
	5	5	3.06		5	5	4.18	
			3.28	0.692[e]			3.49	0.633[e]

[a]Reference: Benzene.

[b]Coefficient of variation: Standard deviation 0/0 mean x 100.

[c]F ratio: Variance ratio between day mean squares and within mean squares.

[d]Not significantly different.

[e]Mean optical density difference for all days.

[f]Significantly different at 1% and 5% level ($P_{4,20}$, 2.89 and 4.43, respectively).

reference using a Gilford 240 Spectrophotometer (Fig. 1). Two absorption peaks were observed, the first at 340 mμ and the second at 410-420 mμ. The increase in sensitivity at 340 mμ was 2.2-fold. In one experiment, the coefficient of variation (CV) at 340 mμ among nine replicates (reagent blanks and cerebroside samples) was 3.36% for cerebroside standards. The same samples when measured at 420 mμ gave higher values both for the reagent blanks (12.02%) and the cerebroside standards (4.70%). In this paper all spectrophotometric assays of the TNBS-sphingosine reaction product were made at 340 mμ.

TABLE II

Effect of 1 M Hydroxide of Hyamine of TNBS-Sphingosine Reaction[a]

Hyamine, ml	Optical density	Mean CV[b]
0	97	33.12
0.01	610	5.02
0.05[a]	654	1.78
0.1	647	4.84
0.5	633	3.28
1.0	4.83	6.11

[a]Total volume 5.5 ml. Reaction conditions as in text with 100 μg brain cerebroside. Quantity of 0.05 ml equals standard assay conditions.

[b]Coefficient of variation.

Experimental error was determined on day to day variation and variation within days (Table I). Benzene was employed as an absolute reference and comparison was made using 100 μg of pure cerebroside. With the test tube procedure (Table I) the mean optical density difference between reagent blanks and samples was 0.692 with an average coefficient of variation of 3.28%. Similar optical density differences, a mean of 0.633 and a coefficient of variation of 3.49%, were observed for the method when assays were made directly on the cerebroside standard when separated by TLC (Table I). The recovery in this experiment was 91.47%. With improvements in the technique of spot detection (waterspraying the TLC plate on a blank background), drying of the labeled TLC plate over magnesium perchlorate, and the use of a blank TLC plate (developed in the same chromatographic solvent), recoveries routinely averaged 95-100%. Recoveries of pure HG.2 and HG.4 ganglioside from Whatman 3 MM paper were 97-98%. The test tube procedure was stable from day to day and showed

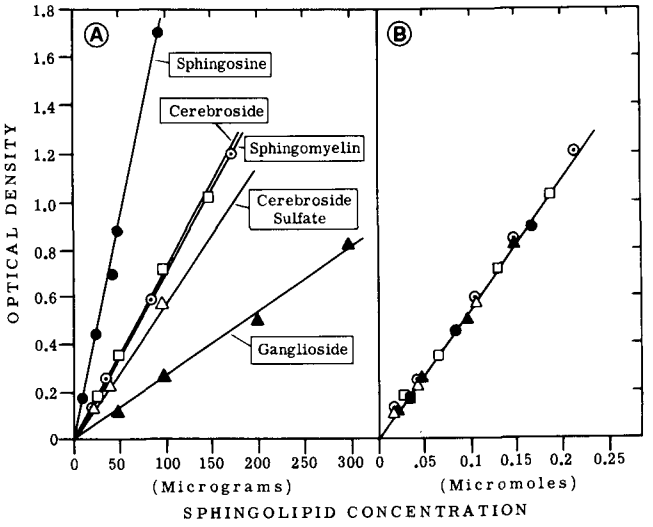

FIG. 2. Concentration-optical density curves for some sphingolipids. A. Data plotted on a weight basis (μg). B. The same data plotted on a molar basis (μM). Molecular weights used for the calculation are given in the text.

minimal variation.

When samples of pure cerebroside were applied to a TLC plate and the assay carried out as indicated for samples containing silicic acid, some deviations were observed (Table I), when compared to samples assayed in the absence of silicic acid (Table I). An analysis of variance did not reveal significant differences between days and within days with the test tube method. The same method as applied to TLC plates revealed some variations for blanks and samples (Table I), but these differences were eliminated when the reagent blanks were subtracted from the samples and only the optical density differences were considered.

The level of Hyamine hydroxide on experimental variability was noteworthy (Table II). Maximum color yield coincided with minimum experimental variation, that is, 2.5% Hyamine v/v of the buffer mixture. Higher concentrations of Hyamine reduced the color yield, increased the reagent blank and increased the coefficient of variation to unacceptable levels.

The optical density data are plotted for various pure sphingolipids, including sphingosine (free base), cerebroside, cerebroside sulfate, sphingomyelin and a ganglioside (HG.2) (Fig. 2). The data are plotted on a weight basis in Figure 2A. The same data are replotted on micromole basis in Figure 2B. The molecular weights for the sphingolipids were calculated using an average fatty acid molecular weight of C_{22} for bovine brain sphingomyelin (molecular wt, 819), bovine cerebroside (molecular wt, 817), bovine cerebroside sulfate, potassium salt (molecular wt, 937). Stearic acid was taken to be the fatty acid in ganglioside, HG.2 (molecular wt, 1965) and ceramide 582.

Optical density weight equivalents relative to cerebroside were obtained for the following sphingolipids: sphingosine (free base), 0.39; sphingomyelin, 0.46; cerebroside, 1.00; cerebroside sulfate, 1.17; and HG.2 ganglioside, 2.72. These data permit the interconversion of optical density readings among the above sphingolipids using cerebroside as a reference, e.g., 0.39 μg sphingosine provides the same optical density as 1.0 μg cerebroside.

The recoveries of three pure sphingolipids from TLC systems is given in Table III. One-dimensional systems provide the highest rates of recovery. Chromatography in two dimensions reduced the recovery of all sphingolipids by 4.86-9.47%. The coefficients of variation also increased after chromatography in the second dimension (1.26% to 8.27%).

DISCUSSION

The absorption spectrum of the TNBS derivative is identical to the spectrum originally observed by Satake et al. (11) for amines and amino acids. Contaminating materials normally present in solvents and TLC media interfere in the 420 mμ region. These interferences are reduced to acceptable levels by confining spectrophotometric measurements in the 340 mμ region. Further reductions in errors were made by employing one vessel for all reaction steps and avoiding multiple extraction, transfer

TABLE III

Quantitation of Sphingolipids Separated by TLC[a]

Sample	Cerebroside	Sulfatide	Sphingomyelin
One-dimensional TLC			
1	96.79	80.42	84.62
2	94.80	91.17	87.34
3	95.55	83.29	88.09
4	96.05	83.77	85.61
	$\bar{x}$ 95.79 ± 0.83	84.66 ± 4.58	86.42 ± 1.58
	CV[c] 0.86%	5.31%	1.82%
Two-dimensional TLC[d]			
1	83.20	85.62	87.05
2	84.50	78.40	76.30
3	91.21	75.40	71.62
	$\bar{x}$ 86.30 ± 4.20	79.80 ± 5.25	78.32 ± 7.91
	CV[c] 4.86%	6.57%	10.09%

[a]Lipids detected as hydrophobic areas with water spray, marked and dried over night in desiccator.

[b]One-dimensional solvent system: chloroform-acetone-methanol-acetic acid-water, 3:4:1:1:0.5 (10).

[c]Coefficient of variation.

[d]Two-dimensional solvent system: chloroform-methanol-ammonium hydroxide 65:25:5; followed by chloroform-acetone-methanol-acetic acid-water, 3:4:1:1:0.5 (10).

and evaporation steps. The presence of small amounts of water in the TLC adsorbent did not interfere with the acylation step with methanolic BF_3; therefore, no special precautions are necessary in drying TLC plates and media. Seven per cent BF_3 was employed in this study because the limiting factor in this reaction is not the concentration of the catalyst (BF_3), but the minimum volume of methanol required to wet the TLC adsorbent or cellulose supporting media-lipid mixture.

When optical density differences were used, experimental variations was within acceptable limits for colorimetric procedures. The technical errors inherent in this method are less than the usual sampling errors in biochemical studies. Losses of pure compounds applied to various chromatographic media were minimal when sensitive detection methods were employed and all transfers of the absorbent were quantitative. Consistent optical density yields per mg for a variety of pure sphingolipid standards were experienced during the 18 months that the procedure was employed.

All sphingolipids assayed provided a linear response to concentration on a weight basis. Differences between individual sphingolipids were eliminated when the optical densities were plotted on a molecular weight basis. Therefore, the method can be applied for the rapid determination of molar ratios of other more complex sphingolipids, including ganglioside mixtures, even though the absolute molecular weights may not be known exactly. Since the method was reproducible from day to day over 18 months, absolute standards are not required for the assay of a wide variety of sphingolipids once their conversion factor is known from a pure reference compound, such as cerebroside.

In the presence of Hyamine hydroxide, a quaternary detergent, sphingosine and related amines are efficiently extracted into organic media. With the Hyamine concentration employed in this study, the reaction of the amine and the TNBS reagent is nearly instantaneous at room temperature. The optimum concentration was 2.5% of the bicarbonate-carbonate buffer mixture. Higher concentrations of hyamine increased solution turbidity, interfering with optical density measurements because of rapid phase changes, and reduced the color yield. Other detergents, such as Triton X-100 or Lubrol WX, were not effective in this assay system.

The principal source of variation in this procedure is the neutralization of the BF_3 catalyst with the bicarbonate-carbonate buffer. The reagent concentrations employed in the described procedure effectively neutralize 1 ml of 7% BF_3 and maintain the pH during the reaction step with TNBS at the optimum for the formation of the amine-TNBS reaction product, pH 7.4-7.8. The buffer mixture is prepared fresh for each day. This mixture sepa-

rates into two phases on standing and should be used as a one-phase system.

Recoveries from TLC were nearly quantitative (Table III). Additional losses and increased variability were experienced when chromatography was carried out in a second dimension. Since the losses increased with each successive chromatography run, reduced yields of sphingolipids were probably the result of "tailing" or adsorption. However, these variations can be corrected for by increasing the number of replicate TLC and correcting back to 100% recovery with percentage recovery data obtained by chromatography and assaying pure sphingolipid samples.

ACKNOWLEDGMENT

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The Composition of Beef Heart Cardiolipins Isolated by Solvent and Chromatographic Fractionation

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ABSTRACT

A method is described for the isolation of cardiolipin from beef heart lipids by a single pass through a silica gel column. The isolated cardiolipin was free of neutral lipids and phospholipids and accounted for 10% of total phospholipid phosphorus. It was compared to commercial cardiolipins prepared by the Pangborn method of selective precipitation. Analysis for fatty acids, glycerol and phosphorus revealed a molar ratio of 2:1.5:1 for both preparations, provided the fatty acid esters were assayed colorimetrically. If the fatty acids were determined by titration, the commercial cardiolipin had a molar ratio of fatty acid to phosphorus of 1.6, while ours remained at 2.0. Upon hydrolysis with acetic acid, the former yielded 76% water-soluble phosphorus, the latter only 2%. Both cardiolipins contained over 90% C-18 fatty acids, with our preparation containing 76% linoleate, the commercial preparation, 90%. After alkaline hydrolyses a component was isolated from the commercial cardiolipin which represented 15.5% of the total weight. It developed an interfering pigment in the fatty acid ester determination but has not been identified. After its removal analysis demonstrated three fatty acids per molecule of the commercial cardiolipin.

INTRODUCTION

Cardiolipin, localized in the mitochondria (1), is a lipid hapten used in various modifications of the Wassermann test for syphilis. It is commercially prepared as described by Pangborn et al. (2) using repeated precipitation of its barium and cadmium salts from methanol. Its isolation by column chromatography has always been difficult and incomplete (3,4). Repeated fractionations to remove impurities have resulted in reduced yields and enhanced peroxidation of the product. Previous investigations on chromatographic adsorbents indicated that Davison silica gels could resolve cardiolipins from other lipids by a single fractionation through a relatively short column (5).

The present investigations describe conditions for the total isolation of cardiolipin from beef heart lipids in very high yields and purity. Its chemical and physical properties are contrasted with beef heart cardiolipin isolated by the Pangborn method. Unexpectedly, the preparations are different. The latter has one less fatty acid and an impurity while the former conforms to the diphosphatidylglycerol structure proposed by MacFarlane and Gray (6).

METHODS

Analytical Methods

Lipid phosphorus was determined by the method of Harris and Popat (7). Acyl groups were analyzed by the method of Snyder and Stephens (8). Fatty acids were analyzed by the method of Dole and Meinertz (9) after hydrolyzing cardiolipin in 0.5 N methanolic potassium hydroxide for 30 min at 40 C. The same method was adapted to titrating the phosphodiester groups in cardiolipin directly without prior hydrolysis. The hydrolysis procedure described above was also used in the determination of the neutralization equivalence of the fatty acids. The hydrolyzed fatty acids were partitioned between hexane and water, titrated and weighed.

Glycerol was determined by the method of Hanahan and Olley (10) as modified by Renkonen (11) and by Courtade et al. (12). Higher chain length aldehydes were determined by the method of Wittenberg et al. (13). Total nitrogen was measured by the procedure of Koch and McMeekin (14) as modified for lipid material by McKibbin and Taylor (15). Hydrolysis with 90% acetic acid was carried out as described by MacFarlane and Wheeldon (16). Chromatography on silica gel loaded paper was performed as described by Marinetti (17). The lipids were detected by examination under UV light after spraying with aqueous Rhodamine 6G (17). The distribution of fatty acids in cardiolipin was determined by conversion to methyl esters with diazomethane (18) and analysis with a Perkin-Elmer Vapor Fractometer #154 using a diethylene glycol succinate column.

The solubility of cardiolipin in acetone or absolute ethanol was determined by adding solvent to the dried material and mixing

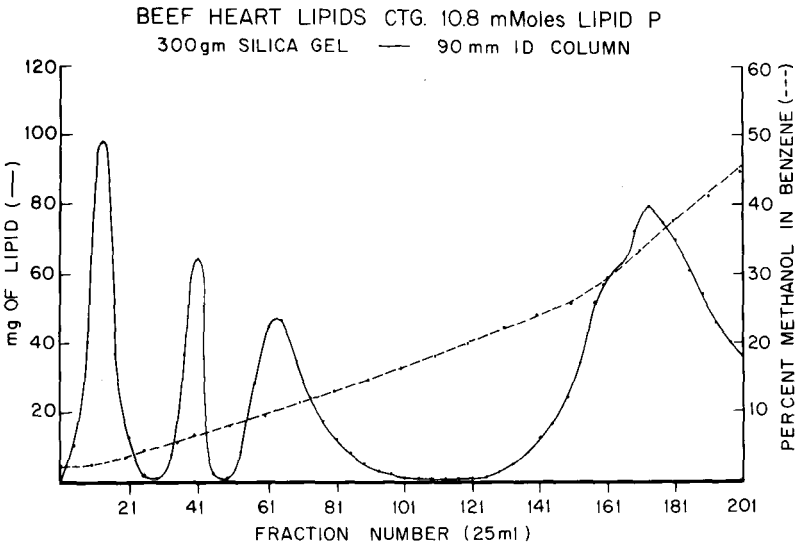

FIG. 1. Composition of peak fractions: first, pigments, cholesterol ester, triglyceride and fatty acid; second, pigment and cholesterol; third, cardiolipin; fourth, phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol.

vigorously in a water bath for 1/2 hour at 40 C. After 6 hr at room temperature with occasional shaking the suspension was centrifuged and an aliquot of the supernatant assayed for phosphorus. Antigenic activity of cardiolipin was determined by the VDRL test for syphilis (19).

Extraction of Beef Heart Lipids

Three hundred thirty grams of lean ventricular tissue from freshly slaughtered beef were minced and then homogenized for 3 min in a Waring Blendor containing 600 ml of chloroform-methanol 1:2 (12). After stirring the homogenate for 1 hr at room temperature, 25 g of Celite (analytical grade) were added, and the slurry filtered through a Buchner funnel. The filtered cake was extracted two more times with 600 ml of chloroform-methanol 1:1 and then 2:1. The total extraction time was about 3 hr.

All extracts were pooled, concentrated under vacuo to about 600 ml and partitioned in a separatory funnel between petroleum ether (30-60 C) and 1% sodium chloride solution. The aqueous methanol phase was reextracted with fresh petroleum ether and the ether extracts pooled and stored in a refrigerator overnight over anhydrous sodium sulfate.

Column Chromatography of Beef Heart Lipids

Davison (Davison Chemical Division, W.R. Grace & Co., Baltimore, Md.) silica gel #62, 100-200 mesh, was prepared in the following three different ways: 1. The gel was washed with methanol and dried in vacuum at ambient temperature. 2. The gel was washed with water and dried at 100 C for 24 hr. 3. The gel was allowed to equilibrate with the laboratory atmosphere for several days and then heated for 15 hr at 72 C. It was then equilibrated in an evacuated desiccator with a beaker containing methanol sufficient to bring the methanol content of the gel to 3.9%.

Three hundred grams of one of the above gels was added to a 90 mm i.d. chromatographic column filled with benzene. A small flow of benzene was allowed to pass through the column during packing. Beef heart lipids dissolved in 35 ml of benzene and equivalent to about 300 mg of phosphorus were placed on the column. The column was attached to a gradient elution apparatus containing 4 liters of 2% methanol in benzene in the reservoir. Bottle and column were connected by siphon as described by Wren (20). The shape of the concentration gradient was determined in a blank run with Sudan IV in the reservoir (Fig. 1). After discarding 460 ml as void volume, 200-25 ml fractions were collected and chromatographed on silica gel paper to locate and assess the purity of cardiolipin. In the text, the isolated cardiolipins are designated beef heart cardiolipin, BHC-1, 2, or 3 depending on the pretreatment of silica gel as described above.

Acetone Precipitation of Cardiolipin

Cardiolipin, equivalent to 2 mg of phosphorus, was dissolved in 1 ml of benzene and

TABLE I

Phosphorus Content (P) of the Cardiolipin Preparations

Preparation			Per cent phosphorus
Commercial cardiolipins			
Beef heart-40 (Sylvana Chemical)			3.98
Beef heart-35 (Sylvana Chemical)			3.92
Beef heart (Pierce Chemical)			3.78
Diphosphatidylglycerol as sodium tetralinoleate, theoretical			4.14
Silica gel column cardiolipins, acetone precipitation			
Beef heart-1	2.68[a]	3.90[b]	99.0[c]
Beef heart-2	3.43[a]	3.51[b]	95.0[c]
Beef heart-3	3.68[a]	3.88[b]	98.5[c]

[a]Per cent phosphorus before acetone precipitation.

[b]Per cent phosphorus after acetone precipitation.

[c]Per cent total lipid phosphorus recovered in the precipitate.

15 ml of dry acetone was added slowly. The contents were briefly swirled in a 40 C water bath and refrigerated overnight at 4 C. The white precipitate was dissolved in benzene-methanol and pooled with other acetone precipitates. The supernatant was concentrated to 1/4 of its volume and stored at -20 C overnight. The very small amount of precipitate formed was combined with the first precipitates. The acetone precipitated cardiolipin was assayed for purity by paper chromatography and by phosphorus content.

RESULTS

Of the three methods described above for pretreatment of silica gel, the third gave the best recovery of cardiolipin. With the large columns employed (90 mm i.d.), the ratio of eluant to adsorbent was limited by the capacity of the gradient reservoirs. Under these conditions complete separation of cardiolipin from neutral lipids could be achieved only by properly deactivating the gel. As shown in Figure 1, cardiolipin was separated as a single peak free of other phospholipids and neutral lipids. Under these conditions 2.2 μmoles of cardiolipin phosphorus was isolated per gram of fresh beef heart or 10.2% of the total lipid phosphorus.

The phosphorus content of the cardiolipin preparations varied somewhat, although paper and column chromatographic evidence indicated but a single component. The cardiolipins

TABLE II

Chemical Analysis of Cardiolipin Preparations

Preparation	Fatty acid ester, μmoles per μatom P	Titratable fatty acid μEq per μatom P	Glycerol, μmoles per μatom P	Titratable acid intact lipid, μEq per μatom P	Per cent water soluble phosphorus after acetic acid hydrolization
Beef heart-1	2.10	1.92	1.46	0.96	1.35
Beef heart-2	2.10	1.89	1.44	0.93	1.05
Beef heart-3	2.14	1.94	1.44	0.99	1.96
Commercial cardiolipin					
Sylvana Chemical-40	2.01	1.61	1.48	0.74	75.0
Sylvana Chemical-35	2.04	1.52	1.45	---	76.8
Pierce Chemical	2.11	1.65	1.50	0.74	76.5
Diphosphatidyl-glycerol[a]	2.00	2.00	1.50	1.00	---

[a]Theoretical value.

were then purified by precipitation from dry acetone. As noted in Table I, from 95% to 99% of the total lipid phosphorus was recovered in the precipitate. The change in phosphorus content after acetone precipitation indicates that some neutral lipid component, associated but not covalently linked to cardiolipin, was eliminated. Table I gives the phosphorus content of several commercial preparations (The Sylvana Company, Millburn, New Jersey 07041, and Pierce Chemical Company, Rockford, Illinois 61105.) isolated by the solvent precipitation method of Pangborn (2).

All cardiolipin preparations were chromatographed on silica gel impregnated paper in neutral (diisobutylketone-tetrahydrofuran-water, 5:40:6), basic (diisobutylketone-pyridine-water, 55:41:4), and acidic (diisobutylketone-acetic acid-water, 40:20:3) solvent. The R_f values of all the preparations were identical. However, chemical analysis revealed some striking differences in chemical composition (Table II). For our cardiolipins, the molar ratios of fatty acid to phosphorus as determined by titration are in close agreement with the fatty ester to phosphorus ratios. On the other hand, the commercial cardiolipins gave titration values significantly below the fatty ester values and lower than theoretical. Subsequent evidence indicated that these possessed a component exhibiting an anomolous ester value.

The molar ratios of glycerol to phosphorus for all preparations were close to the 1.5 ratio, consistent with a diphosphatidyl-glycerol structure (6). Our unhydrolyzed cardiolipins gave the theoretical titratable acid to phosphorus molar ratio of unity whereas the commercial preparations gave only 75% of this. The average molar ratio of fatty aldehyde to phosphorus in the commercial preparations was 0.05 while in our cardiolipins the average value was 0.04. Gray and MacFarlane have reported the absence of plasmologens in beef heart cardiolipins (3).

Not more than 2% of the phosphorus in our cardiolipins became water soluble by hydrolysis with 90% acetic acid, whereas 75% or more was solubilized from the commercial preparations. Extensive hydrolysis of cardiolipin in acetic acid has been reported for beef heart preparations by Faure and Morelec-Coulon (21,22) and by MacFarlane and Wheeldon (16). The latter authors reported release of 83% of total phosphorus as water soluble diphosphoglycerol. Our preparation is therefore unusual in this resistance to acetic acid hydrolysis.

The principal fatty acid in all preparations was linoleic acid and over 90% of the fatty acids were C-18. The per cent by weight of linoleic acid in our cardiolipin, the Sylvana Chemical, and Pierce Chemical cardiolipins was 76.0, 90.5 and 90.0, respectively; of oleic acid, 8.4, 5.7 and 7.5; and the sum of palmitic and palmitoleic acids, 4.1, 2.8 and 2.4, respectively. Our cardiolipin also contained 2.0% stearic acid and 7.9% linolenic acid, neither of which were detected in the commercial cardiolipins. The greater proportion of linoleic acid in the latter may indicate a selective and incomplete isolation or a hydrolytic loss of fatty acids other than linoleic acid in the Pangborn procedure.

TABLE III

Solubility Characteristics of Cardiolipin

Preparation	Solubility of cardiolipin mmoles phosphorus per liter	
	Absolute ethanol	Acetone
Beef heart-3	0.32	0.75
Beef heart, (Sylvana Chemical)	>11.0	>4.36

Table III compares the solubility of the preparations in polar organic solvents. The commercial cardiolipin is 34 times more soluble in absolute ethanol and six times more soluble in acetone than ours, suggesting significant differences in composition.

The neutralization equivalents of the fatty acids are recorded in Table IV. From the distribution of the fatty acids in cardiolipins, the expected neutralization equivalent for either preparation should be approximately 280. The value for the commercial preparation was higher than expected. After centrifuging the hexane-water system containing the products of hydrolysis, a finely divided substance gathered at the interphase. It was removed by ethyl ether extraction and this reduced the neutralization equivalent from 295 to 276. This material migrated on silica gel paper as a single component in the region of phosphatidic acid, yet it contained no phosphorus. Analysis for fatty esters gave an off color purple with an absorbance one sixth the value given by the same weight of methyl linoleate. It absorbed in the UV from 270 mμ to 220 mμ showing increasing absorption at shorter wavelengths but exhibiting no maxima or minima. Recovery of this material was never complete but varied from 5.5% to 15.5% of the commercial cardiolipins.

During the determination of the neutralization equivalents, an aliquot from the same hexane phase was removed for a redetermination of the molar ratio of fatty acid to phos-

TABLE IV

Neutralization Equivalent, Molar and Weight Ratios for Cardiolipin Fatty Acids

Preparation	Neutralization equivalent	Neutralization equivalent, centrifuged	μmoles fatty acid per μatoms phosphorus	Weight of fatty acid per Weight of phosphorus
Beef heart-3	285	285	2.01	18.8
Beef heart (Sylvana Chemical)	295	276	1.66	14.5

phorus. The results are recorded in Table IV and are identical to those found previously for the same preparation (Table II), indicating that the treatment in this analysis had no effect on these values. The weight ratio of fatty acid to phosphorus is 23% lower for the commercial cardiolipin than for ours (Table IV). This reduced value is consonant with the lower molar ratio of titrated fatty acid to phosphorus and with the presence of a unique lipid component in the former.

Both preparations demonstrated reactivity in the VDRL diagnostic test for syphilis, but no attempt was made to differentiate reactivity on the basis of structural differences.

DISCUSSION

The above chromatographic method of isolation is unique in that relatively large amounts of beef heart cardiolipin are obtained under comparatively mild conditions. Separation is effected by a single pass through a short moderately deactivated silica gel column. Irreversible adsorption is minimal. A twofold greater yield is obtained (1.5 g/kg wet weight of tissue or 10.2% of phospholipid phosphorus) than by the selective precipitation method of Pangborn (2), and places cardiolipin among the major phospholipid components of this tissue. Moreover the method avoids the prolonged exposure of the minced tissue to the aqueous acetone-aqueous methanol extraction system and possible artifacts arising from hydrolysis or solvolysis of cardiolipin. Our yield of total phospholipid extracted from this tissue is comparable to that obtained by Mallov et al. (23) using the more polar solvent system of alcohol-ether 3:1.

In our fractionation (Fig. 1) the total tissue cardiolipin emerges as one peak fraction. None is found trailing in other fractions as reported by Shimojo and Ohno (24). The preparation is colorless, free of other phospholipids and the neutral lipids found in heart. However, an acetone soluble component is present in varying amounts. Its presence is not likely to be the result of incomplete elution of the neutral lipids, since it occurs in preparations from which all other neutral lipids have been removed. It may be a product of phospholipid degradation, continuously formed during fractionation. Repeated chromatographic fractionation reduces but does not eliminate this substance. Whatever its origin the substance is closely associated with cardiolipin, since repeated acetone precipitations do not remove it completely.

Irrespective of the variation in phosphorus content from theoretical, our beef heart cardiolipin contains the same molar ratio of fatty ester to glycerol to phosphorus as reported by Faure and Morelec-Coulon (21) and by MacFarlane and Gray (6). On the other hand, the commercial cardiolipins appear to have less esterified fatty acid. The lower molar and weight ratios of fatty acid to phosphorus, the increased solubility in polar organic solvents and the extensive hydrolysis in acetic acid are all consonant with this conclusion.

It is possible that in the Pangborn method fatty acids are lost by hydrolysis during the prolonged extraction period in aqueous acetone-aqueous methanol and that extraction of cardiolipin is actually dependent on this. According to our solubility data (Table III), without the loss of fatty acids and generation of hydroxyl groups, cardiolipin would show sparing solubility in methanol. If the fatty acids were selectively lost from a β-hydroxyl group the extensive hydrolysis in acetic acid could be explained by the mechanism proposed by MacFarlane and Wheeldon (16) and by Coulon-Morelec et al. (22). According to these investigators, a phosphodiester group next to a neighboring hydroxyl group readily forms a phosphotriester in acid solution. The unstable phosphotriester hydrolyzes to give a variety of products depending on which of the ester phosphates is cleaved. Presumably, the extra free

hydroxyl group in cardiolipin prepared by selective precipitation accelerates hydrolysis in acetic acid. The resistance of our cardiolipin may be due to the hydroxyl group in an α-position which cannot participate in the formation of triesterphosphate. All attempts to acetylate or oxidize this hydroxyl group have failed.

Evidence for a keto group instead of a β-hydroxyl group has not been found either, although an absorption maximum at 272 mμ for cardiolipin corresponds to that of the ketone group of acetone. No fatty aldehydes have been found, indicating the absence of plasmalogens in beef heart cardiolipin. Similar results have been reported by Gray and MacFarlane (3). Furthermore, all preparations, whether they approach the theoretical phosphorus content or not, are uniquely resistant to acetic acid. This does not completely rule out the possibility of an unidentified component in cardiolipin.

The commercial cardiolipin, for instance, possesses a unique component or impurity, but has lost approximately one fatty acid. Our cardiolipin, on the other hand, retains four fatty esters but may have lost this component. A firm association of this component with the commercial preparation is evident, but the mode of association has not been worked out. The component may be chemically linked to the diesterphosphate of cardiolipin to form a triesterphosphate. The occurrence of a triesterphosphate linkage has been suggested in rat tissue cardiolipins (4,12). However an equally probable explanation is that the association is a physical one and the component is an impurity which is associated with cardiolipin with sufficient firmness to resist separation after repeated precipitation of the barium and cadmium salts of cardiolipin with alcohol.

Both cardiolipins are resistant to phospholipase hydrolysis and hence differ from the synthetic cardiolipin prepared by DeHass et al. (25). Since the latter investigators present no analytical data, it is not possible to compare the natural and synthetic preparations. Finally, all the isolated cardiolipins are reactive in the VDRL test for syphilis while the synthetic preparation has been reported to be active in the Kolmer test. Hence, at the level of sensitivity that the tests are performed, the results do not reflect the structural differences that apparently exist. Faure and Coulon-Morelec have reported that several derivative structures of cardiolipin are reactive in the Kolmer test for syphilis (26).

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The Biosynthesis of Polyunsaturated Fatty Acids in Plants[1]

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ABSTRACT

This paper is a review of some of the work being done at the author's laboratory. The phospholipids and glycolipids of the alga, *Chlorella vulgaris*, have been implicated in fatty acid transformations such as chain elongation and desaturation. Labeling studies with [^{14}C]acetate have shown that newly synthesized galactosyl glycerides have mainly saturated fatty acids. Subsequent to de novo synthesis, a series of alterations of fatty acid structure takes place within the same glycolipid molecules. The specific incorporation of [^{14}C]oleic acid into *Chlorella* phosphatidyl choline provides a convenient model system for studying the lipid dependent desaturation of oleic to linoleic acid. The inhibitor of fatty acid desaturation, sterculic acid, only inhibits the conversion of oleate into linoleate if added before the precursor fatty acid has been incorporated into a complex lipid. Studies with isomeric monoenoic fatty acids have suggested that there are two enzymes which catalyze the formation of linoleic from oleic acid. One measures the position of the second double bond from the carboxyl group, the other, from the methyl end of the chain. The latter enzyme probably requires the complex lipid substrate.

INTRODUCTION

The objective of our work is to show how, in desaturation, the two hydrogen atoms are released during the reaction, and to examine the catalytic site of the enzymes. This could be done by obtaining the enzymes in soluble form and purifying them. Our efforts in this direction have been mainly confined to animal desaturases. The work described here typifies another approach, working with rather crude enzyme systems but using a variety of tailor-made substrates to probe the specificity of the desaturase reaction.

Whole cell cultures or subcellular preparations of the green alga *Chlorella vulgaris*, were used as our model for higher plants because fatty acid and lipid composition and general lipid metabolism are very similar to plant leaf chloroplasts. However, there are two main differences: *Chlorella* yields subcellular preparations capable of synthesizing polyunsaturated fatty acids (1,2) which no plant leaf chloroplast preparation has done, and it can directly desaturate exogenous stearic acid (3). Stearic acid or stearoyl-CoA could be directly desaturated to oleic acid in a whole range of animal preparations, but no such reaction could be demonstrated in higher plants. Monoenoic fatty acid synthesis in plants, the so called plant pathway, seemed to be fundamentally different from that in animals. The nearest saturated precursor to oleate was myristic acid, 14:0 (4). The idea of the plant pathway was disproved in two ways. (a) In our laboratory a leaf preparation was incubated anaerobically with labeled

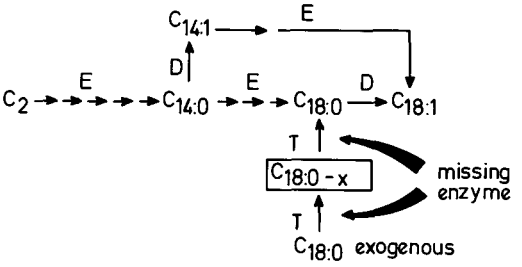

FIG.1. The direct desaturation and plant pathways for oleic acid biosynthesis. X is a hypothetical acyl carrier such as CoA or ACP; E, chain elongation steps; D, desaturation and T, transacylation steps.

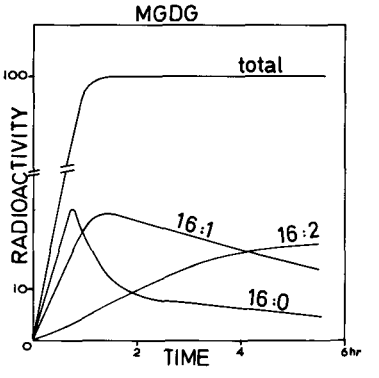

FIG. 2. Fatty acid transformations in monogalactosyl diglyceride (MGDG) subsequent to de novo synthesis from [2-^{14}C]acetate. The ordinate represents the radioactivity of each fatty acid as a percentage of the total radioactivity in MGDG.

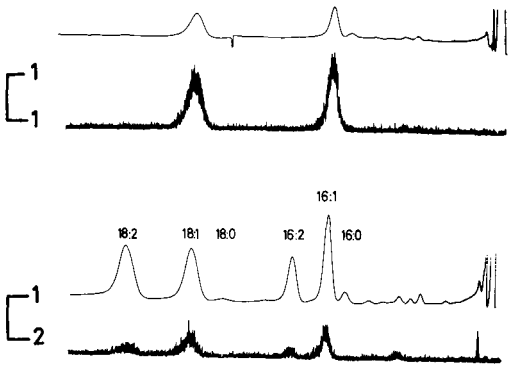

FIG. 3. Differences in the specific activity of monoenoic acids in different MGDG species after incubation with [2-^{14}C]acetate for 2 hr. [1,1] represents species containing two monoenes (two double bonds per molecule); [1,2] represents species containing one monoene and one diene (three double bonds per molecule).

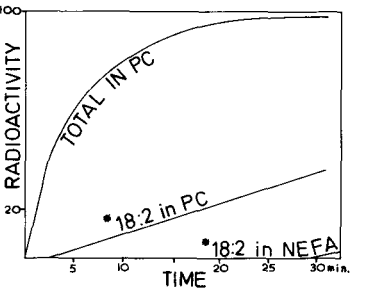

FIG. 4. Time course of incorporation of [1-^{14}C] oleate into PC and its subsequent desaturation to linoleate. PC, phosphatidyl choline; NEFA, nonesterified fatty acid. The ordinate represents radioactivity as a percentage of the maximum label incorporated into PC (about 70% of the added label).

acetate. Labeled stearic acid accumulated and, on transferring the incubation to aerobic conditions, the radioactivity in stearic acid declined, giving place to an equal amount of oleic acid (5). (b) Bloch (6) demonstrated that although spinach chloroplasts would not desaturate stearic acid or stearoyl-CoA, stearoyl-ACP was converted into oleic acid. Thus, plants can perform direct desaturation of stearic acid, and there is no fundamental difference between plant and animal pathways to monoenoic fatty acids. Why then, is endogenous stearic acid itself not desaturated? Bloch's experiments suggest that the substrate must be bound to ACP. We believe that a stearoyl-CoA:ACP acyl transferase is lacking in higher plants. This gives rise to two metabolic states of stearic acid. One is synthesized in situ from acetate and is desaturated to oleic acid without becoming mixed with the other pool which is available to exogenously added stearate. *Chlorella* has proved to be a useful tool because the transferase appears to be inducible by transferring heterotrophically grown cells to autotrophic conditions (Fig. 1) (1).

INCORPORATION OF FATTY ACIDS INTO LIPIDS AND DESATURATION

One cannot study fatty acid synthesis and desaturation without studying the related area of complex lipid synthesis since a cell does not synthesize fatty acids as an end in itself. Because nonesterified fatty acids (NEFA) are extremely inhibitory to most enzymes and must of necessity be maintained at a low concentration, only minute levels are usually found in cells. The object of fatty acid synthesis is to provide the lipophilic moiety of a complex lipid whether that lipid be used as an energy storage compound or a membrane component. This must be kept in mind during the study of fatty acid biosynthesis and desaturation.

In the incorporation of labeled acetate into *Chlorella* lipids (7), all lipids become labeled, but there is a group of three lipids, phosphatidyl glycerol (PG), phosphatidyl choline (PC) and monogalactosyl diglyceride (MGDG) which incorporate radioactivity very rapidly. Once the maximum uptake of label has occurred, the total amount of radioactivity in these lipids remains constant for a considerable time. Initially most of this label is present in the saturated acids 16:0 and 18:0. As the incubation proceeds, even though the total amount of label in a given lipid remains constant, the label in 16:0 and 18:0 falls while the label in the corresponding monoenes first increases and then declines, and so on through to the trienoic acids. Figure 2 illustrates this point using the MGDG and the C_{16} acids as an example.

Another way of looking at this phenomenon is to fractionate the MGDG into individual molecular species (8). When this is done, the specific activities of individual fatty acids vary considerably from one species to another. For example the specific activity of monoenoic acids in species containing two double bonds per molecule is more than six times greater than in species containing three double bonds per molecule after a 2 hr incubation (Fig. 3). This situation can arise if a series of alterations of fatty acid structure takes place within the MGDG molecule subsequent to de novo synthesis from saturated fatty acids. During the course of the incubation these fatty acids are desaturated and the label passes steadily from species of lower degree of desaturation to those more highly unsaturated.

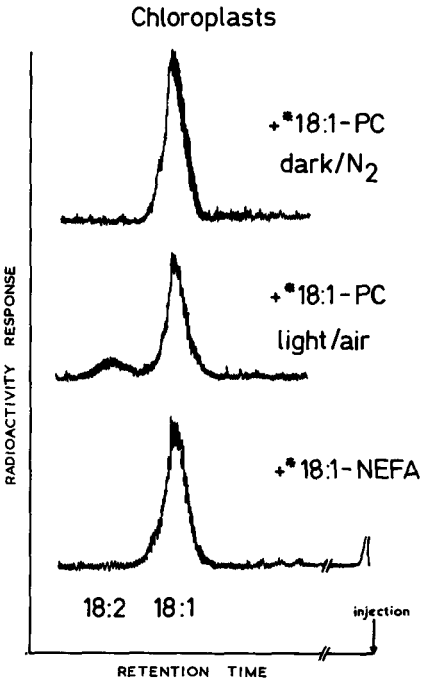

FIG. 5. The conversion of endogenous [^{14}C] oleoyl-PC into [^{14}C] linoleoyl-PC. The diagrams represent radiochemical gas liquid chromatography traces. For the sake of simplicity, mass traces are not shown.

In another type of experiment, ^{14}C-labeled oleic acid rather than acetate was used as precursor (2). Unlike acetate, which is incorporated into most of the phospholipids and glycolipids of *Chlorella*, about 70% of the oleic acid is incorporated extremely rapidly into phosphatidyl choline (Fig. 4).

Desaturation occurs at a slower rate than incorporation, and when it begins after an initial lag, much of the oleic acid has already been incorporated into PC. The linoleic acid which is formed by desaturation is located exclusively in the PC fraction. Labeled linoleic

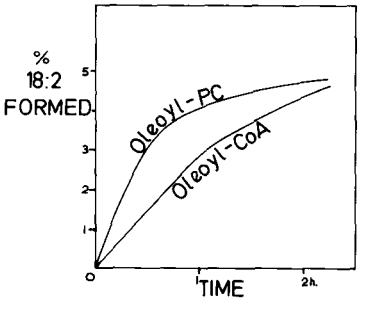

FIG. 6. The time courses for the desaturation of oleoyl-PC and oleoyl-CoA.

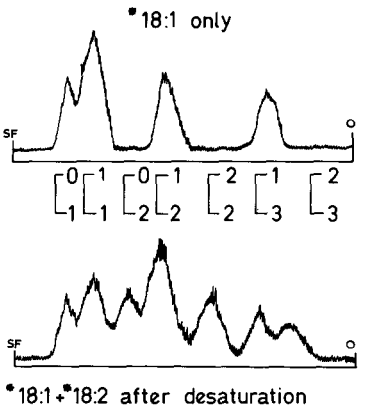

FIG. 7. The distribution of radioactivity among diglyceride species derived from PC. In the upper diagram, the species were derived from cells grown in the presence of [1-^{14}C] oleic acid under dark anaerobic conditions. In the lower diagram, the species were derived from cells grown in the presence of [1-^{14}C] oleic acid under light aerobic conditions. Under these conditions there was an overall conversion of oleic into linoleic acid of 46%. The PC was purified from the lipid extract by DEAE cellulose chromatography, hydrolyzed by phospholipase C and the resulting diglycerides separated by argentation thin layer chromatography in 4% ethanol in chloroform. O, origin; SF, solvent front. The nomenclature for the different species is explained in the legend of Figure 3.

acid is not detected in the NEFA fraction until well after 30 min when a major part of the label has already been incorporated into lipid and desaturated. Experiments such as these suggested that the desaturation of oleic acid was taking place on the lipid. In other words, the lipid is the substrate, or a "carrier molecule" for the desaturase. To test this hypothesis, two types of experiments were performed (2). In the first, PC labeled with ^{14}C-oleic acid was synthesized by growing *Chlorella* in the presence of the labeled acid, in the dark, under an atmosphere of nitrogen, so that no desaturation could take place. After purification, the labeled lipid was sonicated in a buffer to give an almost water clear micellar dispersion which was incubated with a *Chlorella* chloroplast preparation. Linoleoyl-phosphatidyl choline was formed in a yield of 3%. The linoleate could not have come from oleic acid released from the lipid by phospholipase action, because the chloroplast fraction contains no activating enzyme and cannot desaturate NEFA. Although this approach gave us the information that oleoyl-PC is a substrate for the desaturase, it is nevertheless technically very difficult to do this kind of experiment because of problems involved in solubilizing the substrate. In addition, PC micelles, in the required concentration, were inhibitory to the enzyme. Another approach

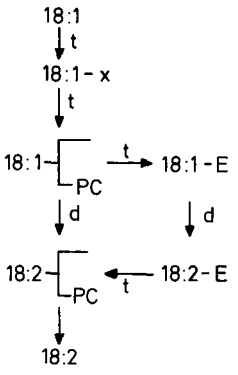

FIG. 8. Hypothetical pathways for the formation of linoleic acid in *Chlorella vulgaris*. The fatty acids are assumed to be incorporated into phosphatidyl choline by the acylation of lysophosphatidyl choline catalyzed by specific acyl transferases.

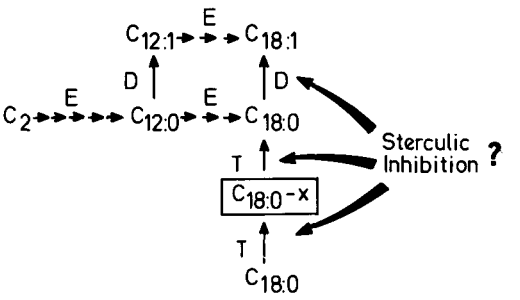

FIG. 9. Different proposals for the site of inhibition of desaturation by sterculic acid. See legend of Figure 1 for explanation of symbols.

was more successful (2).

Chlorella PC was labeled with oleic acid in the dark under N_2 as described. A chloroplast fraction was then prepared and all but a very small amount of unincorporated oleic acid washed out. The result was a desaturase preparation labeled with phosphatidyl choline in situ, but unable to desaturate through lack of oxygen and light. When these preparations were brought into the light and aerated, 10% of oleoyl-PC was transformed into linoleoyl-PC during a 2 hr incubation (Fig. 5). As a final check on this reaction the rate of desaturation of lipid-bound oleate was compared with that of oleoyl-CoA. As Fig. 6 shows, the oleoyl-PC is the better substrate.

Analysis of the molecular species of PC labeled with ^{14}C-oleic acid alone indicated that four of the seven different PC species which contain oleic acid had incorporated the label (Fig. 7). After exposure of cells labeled with oleoyl-PC to desaturating conditions there was a movement of label towards more highly unsaturated species. As in the acetate experiment, the specific activities of oleate and linoleate differed widely in different species. No single species appeared to be involved exclusively in the desaturation reaction, although the species containing two oleic acid molecules has the highest turnover and may be quantitatively the most important species as far as linoleate production is concerned (9).

The current concept of the coupling of oleate desaturation with PC biosynthesis is summarized in Figure 8. One possibility is that the oleoyl-PC acts as an acyl donor to the enzyme, the acid is desaturated to linoleate as an acyl enzyme intermediate, followed by transfer of the acyl group back to PC. The whole reaction would be highly compartmentalized, ensuring that the linoleate remains associated with PC. Alternatively, the fatty acid may remain bound in ester linkage with PC throughout the reaction (2). At present, there is no conclusive evidence distinguishing between these two mechanisms.

TWO POSSIBLE MODES OF DESATURATION

So far, there is less evidence that stearic acid desaturation is as intimately linked with phospholipid or glycolipid synthesis as is oleic acid desaturation. Indeed, nearly all the evidence we have accumulated suggests that the mechanisms for desaturating stearate and oleate are quite different. One of the ways of probing enzyme mechanisms and alternative metabolic routes is by the use of inhibitors. A compound which specifically inhibits desaturation is the cyclopropene acid, sterculic acid:

$$CH_3(CH_2)_7C \overset{CH_2}{=} C - (CH_2)_7COOH$$

Neither the site nor the mechanism of action of sterculic acid is yet known. In relation

TABLE I

Effect of Sterculic Acid on the Desaturation of Oleic Acid

Dark-N_2, hr		Light-air, hr	%
16	1	5	18:2
Chlorella + 18:1	No addition	Transfer	47
Chlorella + 18:1	+ CPE[a]	Transfer	44
Chlorella + 18:1 + CPE		Transfer	19

[a]CPE, Cyclopropene acid (sterculic acid); see text.

TABLE II

Formation of Methylene Interrupted Dienes: Specificity With Regard to Double Bond Position of the Monoene Substrate

Δ9 →Δ9,12-diene	ω9 →ω6,9-diene	Monoenes giving no diene
15:1 ω6	16:1 Δ	Δ7 15:1 ω8
16:1 ω7	16:1 Δ7	Δ7 18:1 ω11
17:1 ω8	17:1 Δ8	Δ11 18:1 ω7
18:1 ω9	18:1 Δ9	Δ8 18:1 ω10
19:1 ω10	19:1 Δ10	Δ10 18:1 ω8
		Δ12 18:1 ω6 → Δ9,12-18:2 by Δ9 desaturase

to *Chlorella*, sterculic acid, at a level twice that of the substrate concentration, inhibits 72% of the desaturation of stearate to oleate, whereas the desaturation of oleate to linoleate is hardly affected (10). Alternatively, a dose 100 times that of the substrate is needed to inhibit oleate desaturation by the same amount, and thus the mechanism of this inhibition appears to be of a different nature from the stearate-oleate inhibition. While the formation of oleic acid from stearic acid is dramatically inhibited by sterculic acid, the inhibition is decreased if labeled acetate is the precursor of oleate. A similar phenomenon is seen in rat (11) or hen (12) liver. Reiser and Johnson believe that the action of sterculic acid is directly on the stearate desaturase enzyme. When this is inhibited by sterculic acid an alternative pathway, according to Reiser, is able to bypass stearate desaturation. This bypass involves desaturation at the C_{12} level (Fig. 9). In our opinion, the inhibition is at the level of an acyl transferase (possibly involved in transferring stearic acid to stearoyl-ACP), which prevents labeled stearic acid from reaching the desaturase complex (Fig. 9) (13). Stearate arising from acetate, however, is coupled directly to the desaturase complex and so bypasses the blocked acyl transferase. Any small inhibition of oleate formation from acetate is probably of a less specific kind. Experiments were made to clarify some of these points.

TABLE III

Effect of Sterculic Acid (CPE) on the Formation of a Dienoic Acid From Δ9-18:1 or Δ12-18:1 Monoenes

	Desaturation, %		
	Control	CPE	Inhibition, %
Δ9	50	46	8
Δ12	7.0	3.3	53

If some desaturations depend on the prior incorporation of the substrate fatty acid into a complex lipid, is it possible that sterculic acid is incorporated preferentially into lipid, effectively blocking the desaturation? Sterculic acid is incorporated at a rate which is equal to, or slightly greater than, oleic acid and very much greater than stearic acid. This might account, therefore, for the relatively large quantities required to inhibit oleate desaturation by a competitive effect for sites on the lipids, but could not explain the more specific effect on stearate desaturation. In another experiment the following operations were carried out: (a) A control incubation consisted of incubating ^{14}C-oleate in the dark under N_2 for 17 hr then transferring the incubation to the light in air for a further 5 hr. (b) The same experiment was done anaerobically for 16 hr. Sterculic acid was then added and incubated a further hour, followed by a light-aerobic phase of 5 hr. (c) Finally, sequence (a) was repeated exactly except that sterculic acid was present from the very beginning (Table I). Only in (c) was there significant inhibition. These results are interpreted to mean that sterculic acid has no effect on oleate desaturation when added after all the labeled precursor has been incorporated into lipids, as it certainly has after 16 hr.

SPECIFICITY OF DESATURASES WITH REGARD TO DOUBLE BOND POSITION

As far as the desaturase which introduces the first double bond is concerned, all saturated fatty acids from 12:0 to 19:0 yield a monoenoic acid with the double bond in the 9,10 position (14). Since the double bond is always in the same position regardless of chain length, we conclude that the active site of the enzyme measures the site for desaturation from the carboxyl end of the molecule. (In *Chlorella* the situation is complicated by the existance of

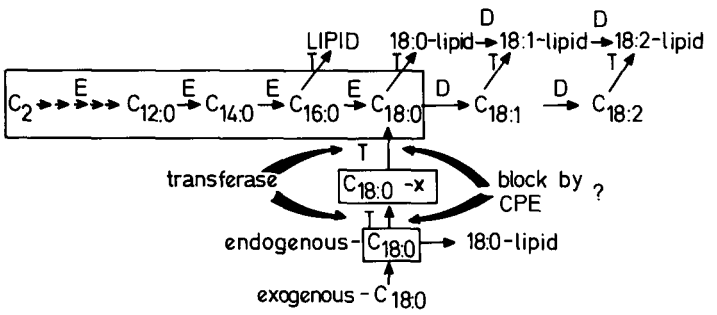

FIG. 10. Interdependent pathways of fatty acid synthesis, desaturation and complex lipid biosynthesis in *Chlorella vulgaris*.

an enzyme which produces a *cis*-7-monoene, but for simplicity this discussion will be confined to the *cis*-9 series).

The question now arises, does the enzyme which introduces the second double bond measure from the carboxyl end of the chain, from the methyl end, or solely with reference to the first double bond? To investigate this, three series of monoenoic fatty acids were synthesized. The members of a given series differed in chain length, but series (a) had the double bond in the carboxyl-9 position, series (b) in the ω-9 position, while in series (c) the bond was neither ω- nor carboxyl-9 (D. Howling et al., in preparation.)

The results of these studies, shown in Table I, are as follows: The chain length of the monoene is not critical within the range C_{15}-C_{19}. Outside this range desaturation is severely restricted. Within this range all Δ9 and ω9 monoenes were desaturated to yield a methylene-interrupted diene. Monoenes with no double bonds in these positions were not substrates, with the exception of Δ12-18:1, which was converted into linoleic acid. The yield of linoleic acid from Δ9-18:1 (ω9, oleic acid) was at least twice that from any other substrate. The interpretation of these results is that there are two enzymes capable of producing a diene from a monoene. Each enzyme will only insert the second double bond to produce a methylene-interrupted fatty acid. One enzyme inserts the second double bond by measuring from the carboxyl group. This is the enzyme responsible for producing *cis,cis*-$\Delta^{9,12}$-dienes from Δ9 monoenes. The other has methyl end control and is the enzyme which produces *cis,cis*-ω,6,9-dienes from ω9 monoenes. Since oleic acid is both a Δ9 and ω9 monoene, it is capable of being desataurated by both enzymes. Exactly the same results have been obtained with a higher plant, the castor bean. Since the carboxyl end is locked up in the lipid while the fatty acid chains project and their ends are available for interaction with the enzyme, it is probable that the lipid-linked desaturase is the methyl end controlled enzyme.

TABLE IV

Tritium-Carbon Ratios of Lipid Components of *Chlorella* After Incubation With [1-^{14}C] Oleic and [9,10^3-H_2] Oleic Acids[a]

	$^3H/_{14}C$
Phospholipids Glycolipids	8.2
Triglyceride	8.9
NEFA	8.9
Substrate	10.0
Diglyceride	10.3
Water soluble	11.3

[a]Mean of 10 values ± s.d. 0.5.

As mentioned earlier, Δ12-18:1 is anomalous in that it is the only monoene which does not have a double bond in the ω9 or Δ9 position, yet produces a diene when incubated with *Chlorella* (Table II). The diene in this case is Δ9,12-18:1, linoleic acid, and the second double bond is in the Δ9 position. The desaturation of this substrate was probably catalyzed by the stearate desaturase, especially as the same reaction is catalyzed by animal preparations which cannot form linoleate from oleate. To check this we made use of the fact that low concentrations of sterculic acid, which have little effect on oleate desaturation, severely inhibit stearate desaturation. We incubated a mixture of tritium-labeled oleic acid with carbon-labeled Δ12-18:1 with and without 1 mM sterculic acid. The results (Table III) indicate that, while the desaturation of oleic acid was inhibited only 8%, the desaturation of Δ12-18:1 was inhibited 53%. This is consistent with the view that linoleic acid is formed from

Δ12-18:1 by the stearate desaturase. Although these two substrates behaved differently as far as desaturation was concerned, their distribution among *Chlorella* lipids at the end of the incubation was rather similar.

ISOTOPE DISCRIMINATION

When we compared the incorporation of [^{3}H]Δ9-18:1 and [^{14}C]Δ12-18:1, we also ran a parallel incubation using a mixture of tritiated and ^{14}C-labeled Δ9-18:1. The incorporation of these two different forms of oleic acid into the different lipids of *Chlorella* was studied by measuring the tritium-carbon ratio in each individual lipid. The conditions were such that no 18:2 formation took place. The results are shown in Table IV.

The tritium-carbon ratio in the phospholipids, glycolipids and triglycerides is lower than that of the substrate, but higher in the case of the diglycerides and water soluble radioactivity, presumably unincorporated fatty acid or thiol esters. This indicates a preferential incorporation of ^{14}C-substrate into the complex lipids, leaving the residual NEFA enriched in the tritium-labeled substrate. A similar phenomenon has been observed in the case of the incorporation of stearic acids into hen liver lipids. The explanation is not clear at present, but is probably due to differences in the strengths of binding of the acyl chain to the transferase enzyme. The presence of two methylene groups containing pairs of tritium atoms instead of hydrogen atoms may significantly alter the binding energy of the enzyme and substrate. The enzyme reaction is sufficiently sensitive to these small changes for the effect to be reflected in differences in incorporation of the two substrates.

In conclusion, these results are summarized from many types of experiments, and a coherent picture which can be fitted into an overall scheme of fatty acid biosynthesis in plants is given (Fig. 10).

Polyunsaturated C_{18} fatty acids arise by sequential desaturation of oleic acid, which in turn arises, as we have shown, in plants as well as animals by direct desaturation of stearic acid. Normally stearic acid is the end-product of the fatty acid synthetase. Exogenously added stearic acid must be transferred to the central enzyme complex in order to be desaturated. It is this transfer step that may be the target for sterculic acid inhibition of stearate desaturation. The enzyme or enzymes which perform this desaturation fix the substrate at the carboxyl group and the active site is so placed in relation to this binding site that the double bond is inserted at the 9 and 10 positions. We believe that the stearic acid is in a bent conformation on the enzyme surface to bring the two D-hydrogens in the correct configuration for desaturation. It is interesting to speculate that at some time in evolution the Δ7 desaturase arose by deletion of a single amino acid from the relevant part of the desaturase polypeptide chain, which we calculate would bring the active site in the correct position to produce a Δ7 monoene.

There are two enzymes which produce the dienoic acids. One controls the double bond position from the carboxyl group, the other controls this position from the methyl end. This may produce not only some linoleic acid from oleate but also 7,10-diene from Δ7-16:1 (significantly, Δ7-18:1 is not a substrate). Linoleate, therefore, arises from the combined effects of two enzymes. At least one of these enzymes acts on lipid-bound oleic acid and this may be the one which locates the double bond from the methyl end of the chain. Two monoenes are potential precursors of linoleic acid when incubated with *Chlorella*, and by studying their desaturation, differences between two enzymes, i.e., the monoene-forming and diene-forming desaturases, can be pinpointed. Oleic acid, (Δ9-18:1,) is desaturated in the lipid-bound form by the methyl-end diene-forming enzyme whereas Δ12-18:1 is desaturated by the monoene-forming enzyme.

The systems for forming unsaturated fatty acids in *Chlorella* are very complex, despite the simple fatty acid composition of the organism. Transacylases are very important and inseparable from the desaturase systems because some desaturations require lipid-bound substrates. We are aware that many of our observations are open to one or even several other interpretations. Many loose ends and even fundamental points remain to be clarified. Does sterculic acid block the transacylase as we have suggested or does it in fact directly inhibit the desaturase? What are the roles of the lipid-linked and nonlipid-linked systems? Do the same considerations apply to formation of trienoic acids? We hope that current and future researches will supply some of the answers.

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Inhibition of Fat Cell Lipolysis by Low FFA to Albumin Ratios

ABSTRACT

Epididymal fat cells isolated from chronically cold-exposed rats were used to study the inhibition of lipolysis with albumin bound FFA. It was demonstrated that lipolysis becomes inhibited as the class 2 binding sites on albumin are filled and that 50% inhibition occurs at free fatty acid (FFA) to albumin ratios ($\bar{v}$) between 4.6 and 4.0. Experiments with dibutyryl adenosine cyclic phosphate suggest that this is an effect on the fat cell lipases rather than the lipase activating system or phosphodiesterase. The results indicate that reesterification proceeds as a relatively constant percentage of the rate of lipolysis and, therefore, in the absence of exogenous FFA, conditions that reduce lipolysis reduce reesterification. On the other hand, high rates of reesterification can apparently be stimulated even when the class 1 binding sites on albumin are not filled.

INTRODUCTION

Steinberg (1) has reported that the uptake and oxidation of free fatty acids (FFA) is dependent on their average number ($\bar{v}$) bound per albumin molecule. In a system containing calcium, a known FFA acceptor (2), Rodbell (3) has shown that FFA to albumin ratios above 6 to 7 can inhibit ACTH stimulated lipolysis in isolated fat cells. It is not known, however, whether gradual reductions in the value of $\bar{v}$ below 6 can effect the tissue lipase or its noradrenaline activated system. Noradrenaline stimulated glyceride fatty acid (GFA) release is greater (4) in fat cells isolated from chronically cold-exposed than control rats (855 vs 411 μmoles GFA per 1 μg DNA per 30 min). Thus these adipocytes from cold-exposed rats provided a much more sensitive system for examining how low values of $\bar{v}$ affected adipose tissue lipolysis and reesterification.

EXPERIMENTAL PROCEDURES

Animals

Male, random bred albino rats (Charles River Breeding Laboratories, Inc., Wilmington, Mass.) weighing between 50 and 75 g were maintained (3) in a cold chamber at 5 ± 1 C for six weeks prior to killing.

Fat Cell Preparation

Epididymal fat cells were prepared by a modification of the procedure of Rodbell (4,5).

TABLE I

Effect of DBcAMP on Fat Cell[a] Lipolysis and Reesterification

DBcAMP, μm/ml	Lipolysis, mμm GFA/ μg DNA/30 min	Reesterification, mμm GFA/ μg DNA/30 min	Per cent reesterification	$\bar{v}$ Final[b]
0.5	95 ± 7[b]	29 ± 16	31 ± 17	0.44 ± 0.03
1.0	413 ± 30	118 ± 24	29 ± 5 (NS)[d]	0.77 ± 0.01
2.0	546 ± 29	155 ± 20	28 ± 2 (NS)	0.93 ± 0.02
5.0	601 ± 21	207 ± 33	34 ± 4 (NS)	0.92 ± 0.03
10.0	596 ± 13	205 ± 15	(NS)	0.92 ± 0.03

[a]Adipocyte DNA ranged from 3.4 to 4.1 μg per vial.

[b]Initial value of $\bar{v}$ for adipocytes plus albumin equals 0.34.

[c]Each value represents the mean ± S.D. for three experimental replications.

[d]Values for per cent reesterification are not significantly different (NS) from each other.

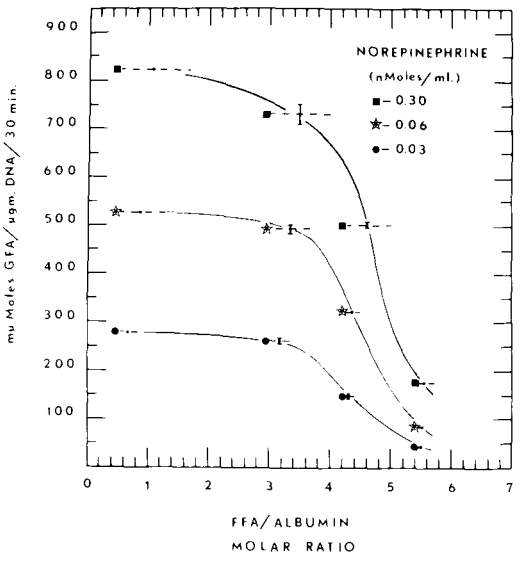

FIG. 1. The effect of increasing values of $\bar{v}$ on noradrenaline stimulated lipolysis in fat cells isolated from chronically cold-exposed rats. GFA (glyceride fatty acid) release equals glycerol production times 3. Symbols also indicate initial values of $\bar{v}$. Dashed lines represent the theoretical increase in $\bar{v}$ as a result of lipolysis (uncorrected for reesterification). Vertical lines denote the standard deviation (when measurable). Each point represents three experimental replications. Adipocyte DNA ranged from 3.6 to 4.1 μg per vial.

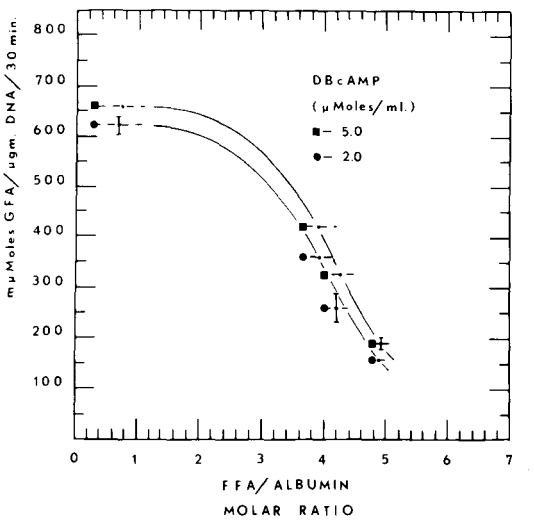

FIG. 2. The effect of increasing values of $\bar{v}$ on DBcAMP stimulated lipolysis in fat cells isolated from chronically cold-exposed rats. GFA release equals glycerol production times 3. Symbols also indicated initial values of $\bar{v}$. Dashed lines represent the theoretical increase in $\bar{v}$ as a result of lipolysis (uncorrected for reesterification). Vertical lines denote the standard deviation (when measurable). Each point represents three experimental replications. Adipocyte DNA ranged from 3.1 to 3.5 μg per vial.

For each experiment, the isolated washed adipocytes of five to seven rats were pooled and stock suspensions diluted to approximately 4 μg fat cell DNA per 1 ml of Krebs-Ringer phosphate buffer containing bovine serum albumin (4%), glucose (1 mg/ml) and no calcium.

Preparation of Albumin Bound Fatty Acid

Stock solutions of albumin bound fatty acids were prepared by combining 1.54 mmoles oleic acid (Fisher, USP) with 50 ml 8% albumin (Pentex, Lot 55) in Krebs-Ringer phosphate buffer at pH 10.5. The mixture was stirred for 1 hr in a 36 C oven, adjusted to pH 7.4 with HCl, and brought to 100 ml with additional buffer. The stock solution was then serially filtered through 0.65, 0.45, and 0.22 μ membrane filters (Millipore Corp.) and the exact FFA content determined by the method of Rodbell (5). The low but measurable fatty acid content of the stock cell suspension and the buffered 4% albumin used as a diluent were similarly determined. These values permitted the calculation of initial FFA to albumin ratios for all dilutions of the adipocyte and bound fatty acid stocks.

The additions of hormones and cells, as well as the incubation procedure, were carried out as previously described (6). After 30 min of incubation, the reaction was terminated by the addition of Doles acid extracting medium and the contents of each vial were assayed for fat cell triglyceride and glycerol production (6).

RESULTS

The effect of increasing FFA to albumin ratios ($\bar{v}$) on the norepinephrine stimulated lipolysis of fat cells in plotted in Figure 1.

Unstimulated lipolysis was approximately 10 mμmole GFA per 1 μg DNA per 30 min and this value, therefore, was arbitrarily assigned as 100% inhibition. This assumption tends to bias the calculation of per cent inhibition towards higher FFA to albumin ratios at any given degree of inhibition. Under these conditions and independent of the norepinephrine concentration used, there was between 80% and 90% inhibition of the observed maximally stimulated lipolysis at FFA to albumin ratios between 5.4 and 5.6. Values of $\bar{v}$ between 4.6 and 5.0 resulted in 50% inhibition of lipolysis. Similarly (Fig. 2), when lipolysis was stimulated with N^6, O^2-dibutyryl adenosine-3′-5′-cyclic phosphate (DBcAMP), 50% inhibition of lipolysis occurred at values of $\bar{v}$ between 4-4.2.

Using fatty acid-poor albumin ($\bar{v}$ = 0.28) without oleic acid, maximum DBcAMP stimula-

tion of lipolysis and reesterification were measured at FFA to albumin ratios less than 1 (Table I).

The maximum observed rate of DBcAMP stimulated reesterification agrees well with that published for norepinephrine stimulation under similar conditions (4). This rate of reesterification, if maintained in all the cell preparations containing exogenous oleic acid (Fig. 1 and 2), would lower the value of $\bar{v}$ at each point by approximately 0.3 in 30 min.

If norepinephrine stimulated lipolysis were inhibited by FFA interfering in the endogenous production of cAMP, then at any given value of $\bar{v}$ (Fig. 1 and 2), we would have found less inhibition with exogenous DBcAMP. This was not the case. On the other hand, if the FFA were activating a phosphodiesterase breaking down cAMP, then two different concentrations of DBcAMP (Fig. 2) would differ in the degree of stimulation at higher values of $\bar{v}$. This, also, was not the case. These results, therefore, indicate that the FFA may be acting directly on the lipolytic enzymes in a concentration dependent manner.

The data in Table I suggest that reesterification proceeds as a relatively constant percentage of the rate of lipolysis in the presence of lipolytic hormone, glucose, but no added insulin. We estimate, also, that the rates of lipolysis noted here are from 2- to 10-fold greater than those obtained with perfusions of adipose tissue (7,8).

Thus, these results suggest that (a) lipolysis becomes inhibited as the class 2 binding sites (9) are filled; (b) even when the class 1 sites are not filled, reesterification may be maximally stimulated; and (c) in the absence of high external concentrations of FFA, conditions that reduce the rate of lipolysis can reduce the rate of reesterification. On the other hand, since we could not accurately determine FFA release in the presence of high levels of exogenous oleic acid (Fig. 1 and 2), it remains an open question whether reesterification was reduced concomitantly with lipolysis in Table I or whether it was increased by the increasing FFA to albumin ratios. It seems likely, however, that under conditions where the transport of endogenous acids out of the cells might be inhibited, reesterification would take precedence, and a greater proportion of the FFA produced would be reesterified.

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Serum Lipoprotein Hydrolysis by Purified Lipases

ABSTRACT

Purified lipases from pork pancreas, *Rhizopus arrhizus* and *Leptospira biflexa* hydrolyze the triglycerides of pork very low density lipoproteins (VLDL), while higher density lipoproteins are not affected significantly. The velocity versus substrate curves for the VLDL were sigmoid.

INTRODUCTION

Lipase (EC 3.1.1.3) hydrolysis of triglycerides in the form of emulsions, mixed micelles, or solutions has been extensively studied (1,2). There are also reports of lipase activity against chylomicrons or total serum lipids (3). Lipoprotein lipase appears to require plasma lipoproteins as cofactors in the hydrolysis of triglycerides (4), while higher concentrations of

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TABLE I

Hydrolysis as Percent of TG Hydrolysis

Enzyme[a]	Triglyceride emulsion	Porcine VLDL	Porcine LDL	Porcine HDL
Pork pancreas	100	28	< 2	<0.1
Rhizopus arrhizus	100	13.5	< 2	<0.1
Leptospira biflexa (Patoc I)	100	45	10	<0.1

[a]Twelve units of enzyme activity as measured against synthetic triglyceride emulsion (14). Experimental conditions (pH, temperature, activators, etc.) as in legend to Figure 1.

plasma low density lipoproteins have been shown to inhibit lipoprotein lipase activity on triglycerides (5). However, little is known concerning the action of lipases on the lipids present in relatively purified lipoproteins. Considering that lipids, transported in the circulation of animals in the form of lipoproteins, are apparently the natural substrates for lipoprotein lipase (6), it appeared of interest to investigate the hydrolysis of the different ultracentrifugal groups of serum lipoproteins by previously characterized triglyceride lipases.

MATERIALS AND METHODS

Lipoproteins were isolated ultracentrifugally from hog serum by a modification (7) of the classical procedure of Havel et al. (8). Protein was determined by the Lowry procedure (9), and triglycerides by the method of Van Handel (10). Three enzymes were investigated: the purified porcine pancreatic lipase (11), the purified extracellular lipase of *Rhizopus arrhizus* (12), and a partially purified extracellular lipase from *Leptospira biflexa* (Patoc I) (13). The extent and rate of lipid hydrolysis was followed by potentiometric titration of the fatty acids, liberated from the substrate, using a TTT-1 pH-stat (14).

RESULTS

In preliminary experiments it was observed that of the three classes of hog plasma lipoproteins isolated: VLDL (very low density lipoprotein, $d < 1.006$), LDL (low density lipoprotein, $1.006 < d < 1.070$) and HDL (high density lipoprotein, $1.070 < d < 1.210$), only VLDL lipids were significantly hydrolyzed by the enzymes studied, Table I. Experiments measuring the effects of systematic variation of pH and reaction temperature, and of the concentrations of NaCl, $CaCl_2$, and the deoxycholate used as an enzyme activator (1,11,12) on the hydrolysis of VLDL by each of the enzymes, resulted in the selection of optimal experimental conditions for the hydrolysis of VLDL by each enzyme which are shown in Figure 1.

The rates of reaction of each enzyme against varying concentrations of VLDL were measured. The data, graphically expressed in Figure 1, indicate that there is significant substrate inhibition of all of the enzymes at elevated substrate levels. At lower substrate concentrations all three curves exhibit a certain degree of sigmoid shape, most pronounced for the pancreatic lipase and least for the *Rhizopus*

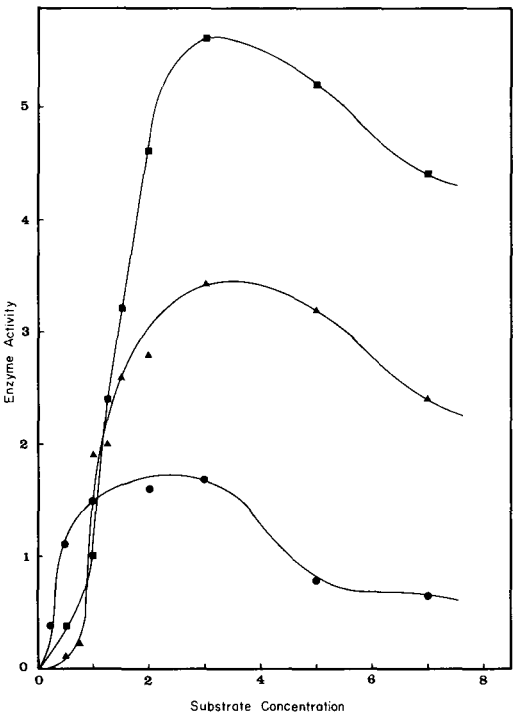

FIG. 1. Variation in lipase activity with VLDL concentration. Enzyme activity, in enzyme units (10^{-6} M fatty acid released per minute). Substrate concentration, in milliliter VLDL (2.0 mg triglyceride per milliliter). (-●-) *Rhizopus* lipase T = 25 C; pH 8.5; Deoxycholate, 10.6 mM; NaCl, 0; $CaCl_2$, 1 mM. (-▲-) Porcine pancreatic lipase T = 35 C; pH 8.5; Deoxycholate, 10.6 mM; NaCl, 0.4 M; $CaCl_2$, 2 mM. (-■-) Leptospiral lipase T = 35 C; pH 8.5; Deoxycholate, 10.6 mM; NaCl, 0.4 M; $CaCl_2$, 2 mM.

enzyme. Furthermore, although amounts of each enzyme equal to 12 units (1 unit = 10^{-6} M fatty acid liberated per minute) as measured against a standard triglyceride emulsion (11) were used, there were significant differences in the maximal activity towards VLDL lipid, the leptospiral lipase being nearly twice as active as the pancreatic enzyme and three times as active as the *Rhizopus* lipase.

DISCUSSION

The results indicate that the three triglyceride lipases studied, presumably typical of this group of enzymes, are able to liberate free fatty acids by the hydrolysis of the complex lipids of porcine plasma VLDL, although at significantly different rates. Inhibition of enzyme activity at high concentrations of substrate, apparent with VLDL, is not observed when triglycerides in emulsion (1) or soluble (2) form serve as substrates. The sigmoid shape of the curves at low VLDL concentration is not observed with triglyceride emulsions (1), but is a characteristic of the hydrolysis of soluble, low molecular weight, triglycerides (2), in which case there is an inflection point which coincides with the critical micellar concentration at which the hydrolysis rate increases markedly over a very small change in substrate concentration. It is interesting to speculate that the physical state, i.e., degree of aggregation, of the complex substrate may be responsible for the sigmoid shape of the curves in our experiments. Some support for this idea comes from reports of subunit structure in lipoproteins (15) and even more from reports of reversible stepwise aggregation of plasma lipoproteins (16). Studies currently under way in this laboratory, on the concentration dependent molecular weight of the plasma lipoproteins and also the effects of surface active agents such as sodium deoxycholate on their state of aggregation, should prove valuable in elucidating the mechanism of this effect.

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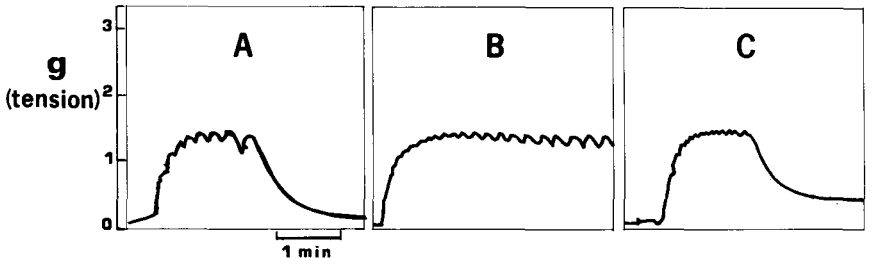

FIG. 1. A, Response of quinea pig uterine horns to 3 m U of pitressin. B, Potentiation effect of 50 ng of standard PGE_1 on the uterine response to pitressin. C, Potentiation effect of about 10 ng of PGE_1 isolated from rat seminal vesicle glands, on the uterine response to pitressin.

demonstrated in human seminal plasma (3,4). In this communication, we describe the isolation of PGE_1 from rat vesicle gland by solvent partition and thin layer chromatographic procedures, its identification by biological assay, and its quantitative determination by spectrophotometry.

Two experiments were performed using 120 and 42 rats respectively. In a third experiment, with 45 rats, tritiated Prostaglandin E_1 (10 μC) was added to the vesicle gland homogenate and its recovery determined. Adult Wistar rats were killed by decapitation. The vesicle glands were removed, homogenized in a Waring Blendor at 4 C in four volumes of ethanol, and centrifuged for 10 min at 3000 x *g* in a Sorval refrigerated centriguge.

The supernatant was evaporated to dryness under reduced pressure, and the residue was dissolved in 0.1 M phosphate buffer, pH 7.0. This solution was washed twice with an equal volume of petroleum ether, first at pH 7.0 and then at pH 3.0, to remove nonprostaglandin lipids (5); the prostaglandins were then extracted from the remaining solution with diethyl ether at pH 3.0. The ethereal solution was finally washed with water to neutral pH and evaporated to dryness under reduced pressure.

The residue was taken up in a small volume of ethanol, spotted on thin layer plates of Silica gel G, and developed in chloroform-methanol-glacial acetic acid-water (90:6:1:0.75 v/v). (6). A standard was used to characterize the R_f of PGE_1. The reference sample of PGE_1 was detected by spraying with 2,4-dinitrophenylhydrazine, keeping the remainder of the plate covered. The zone corresponding to that of the prostaglandin standard was next scraped off the plate and eluted with methanol according to Green and Samuelson (7).

The methanolic solution was evaporated to dryness in vacuo and the residue was dissolved in a small volume of ethanol.

The ethanolic solution was rechromatographed on Silica gel G impregnated with 10% $AgNO_3$, using the solvent system "A II"--ethyl acetate-acetic acid-methanol-2,2,4-trimethyl pentane-water (110:30:35:10:100) (7). Standards for PGE_1 and PGE_2 were used, and their positions on the plate were visualized by spraying with 2,4-dinitrophenyldrazine, while the central region of the plate, with the sample, was covered with a glass plate.

The zones corresponding to that of prostaglandin E_1 (R_f:0.87) and prostaglandin E_2 (R_f:0.77) were scraped off the plate and eluted with ethanol.

An aliquot of the extracts was treated with 2 N KOH in ethanol and heated at 50 C for 25 min. The chromophore was measured at 278 mμ in a Zeiss spectrophotometer and the amounts of PGE_1 and PGE_2 were determined from standard curves (8).

The biological assay was performed according to Clegg et al. (9,10) on uterine horns from nulliparous guinea pigs at 20-22 C in a modified Krebs bicarbonate saline solution, gassed with 5% Co_2 in 95% O_2 and containing 1 mM Ca^{++}, 3.5 mM Mg^{++} and 5.9 mM K^+ in a volume of 3 ml. The potentiation effect of prostaglandin of the uterine response to vasopressin was registered (Fig. 1).

The radioactivity recovered from the plate was measured on aliquots of the ethanolic extract in a Packard Tricarb Liquid Scintillation Counter.

Biological activity was only found present in the ethanolic extracts corresponding to the PGE_1 zone and it is related to a measured PGE_1 content of 0.5 μg/g of wet tissue. PGE_2 was not present in substantial amounts. The final recovery of PGE_1 from rat vesicle gland was about 30%.

In a recent paper, Jouvenaz et al. (11), using a gas chromatography sensitive method, were able to detect amounts of prostaglandin as low as 1 ng in rat tissues. A PGE_2 content of 160 ng/g of rat vesicular gland was measured after homogenization in aqueous medium, but,

apparently, the PGE_2 found is formed during this process by enzymic reaction. This did not occur in our case in which the vesicular glands were homogenized directly in alcohol, thus preventing that reaction. On the other hand, no PGE_1 could be detected, according to the authors, using this new sensitive method.

Our results indicate the presence of PGE_1 in rat seminal vesicle gland. This finding is consistent with the observed in vitro biosynthesis of PGE_1 from homo-γ-linolenic acid in rat vesicle gland (to be published).

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Isomers of Alpha-Tocopheryl Acetate and Their Biological Activity[1]

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ABSTRACT

α-Tocopheryl acetate, an ester of the naturally occurring form of vitamin E, has a biopotency of 1.66 IU/mg as determined by bioassays based on biological function. On a similar basis, 2L-α-tocopheryl acetate, the 2-epimer of α-tocopheryl acetate, has a biopotency of 0.35 IU/mg. Results of hemolysis bioassays indicate relative activities of 1.53 and 0.56 for α- and 2L-α-tocopheryl acetates, respectively, compared with 2DL- or all-*rac*-α-tocopheryl acetate. Responses to plasma and liver storage tests average 1.35 for α-tocopheryl acetate compared with all-*rac*-α-tocopheryl acetate. The configuration at C-2 is most important but the configurations at C-4′ and C-8′ may also be important in determining biopotency. There is no evidence of synergism between α-tocopheryl acetate and 2L-α-tocopheryl acetate. Since the two isomers comprising 2DL-α-tocopheryl acetate, the former International Standard, have different rates of absorption, tissue storage, metabolism and excretion, a better standard for vitamin E is needed. α-Tocopheryl acetate has both the chemical and biological properties required for a new International Standard for vitamin E. Redefinition of an International Unit in terms of 1 IU = 0.60 mg of α-tocopheryl acetate would give a precise reference for future research and a reasonable correlation with data collected in the past.

INTRODUCTION

α-Tocopherol, 5,7,8-trimethyltocol, is biologically the most active member of the naturally occurring tocopherols and tocotrienols. It is a polyisoprenoid derivative characterized by a tetramethyl-6-chromanol nucleus and a saturated C-16 side chain with centers of asymmetry at the 2, 4′ and 8′ positions (Fig. 1). The other tocopherols and tocotrienols have biological activities ranging from 0% to 50% that of α-tocopherol. The acetate esters of the tocopherols have been widely used for both scientific and commercial purposes. This review will describe the structures of the various diastereoisomers of α-tocopherol and their nomenclature and compare the biological activities of the corresponding acetate esters.

NOMENCLATURE

The nomenclature of the tocopherols as recently clarified by the Committee on Nomenclature of the International Union of Nutritional Sciences (1) will be used throughout this review[3]. The name "alpha-tocopherol" is used for the compound, identical to the isomer isolated from natural sources, characterized as 2*D*,4′*D*,8′*D*-α-tocopherol (2R,4′R,8′R-α-tocopherol) and formerly named *d*-alpha-tocopherol (1). Its isomeric structure is detailed in Figure 1. Tocopherols can also be prepared synthetically. The condensation product of trimethylhydroquinone and natural phytol is epimeric only at the 2 position. This equimolar mixture of alpha-tocopherol and its 2 epimer is characterized as 2*DL*,4′*D*,8′*D*-α-tocopherol (2R,4′R,8′R + 2S,4′R,8′R-α-tocopherol) and has been named 2*DL*-α-tocopherol (1). This mixture has been resolved into the two epimeric forms (2), alpha-tocopherol, identical with the naturally occurring isomer, and an unnatural isomer characterized as 2*L*,4′*D*,8′*D*-α-tocopherol (2S,4′R,8′R-α-tocopherol) and named 2*L*-α-tocopherol (1). The condensation product of trimethylhydroquinone and racemic isophytol is a mixture of the four possible enantiomeric pairs of diastereoisomers (8 isomers in all). It is characterized as 2*DL*,4′*DL*,8′*DL*-α-tocopherol and the equimolar mixture has been named *all-rac*-α-tocopherol (1). Formerly, it was termed *dl*-alpha-tocopherol and has often been confused with 2*DL*-α-tocopherol.

---

[1]One of six papers to be published from the Symposium "Chemistry and Biochemistry of Tocopherols," presented at the ISF-AOCS Congress, Chicago, September 1970.

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[3]The Editors of Lipids have not yet accepted the revised IUNS nomenclature system. Except for the section on "Nomenclature", the Editors used the terminology D, L or DL and all-*rac* or All-rac in this paper. This nomenclature system is inconsistent with the revised IUNS, AIN or IUPAC recommendations.

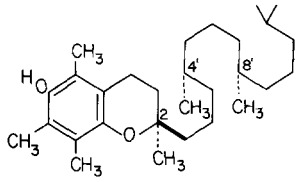

FIG. 1. α-Tocopherol, (*d*-α-tocopherol), (2*D*,4′*D*,8′*D*-α-tocopherol).

STANDARDS FOR VITAMIN E

In 1941, the Vitamin E Sub-Committee of the Accessory Food Factors Committee (Lister Institute and Medical Research Council) recommended "the adoption of synthetic racemic α-tocopheryl acetate as the international standard for vitamin E" and further "recommended that the international unit for vitamin E should be defined as the specific activity of 1 mg of the standard preparation, this quantity being the average amount which, when administered orally, prevents resorption gestation in rats deprived of vitamin E" (3). The International Standard was formerly issued in the form of a 1% solution in olive oil (3) until its distribution was discontinued in 1956 (4). The failure of the sub-committee to identify in detail the isomeric composition of the International Standard for vitamin E has resulted in subsequent confusion. This "synthetic racemic α-tocopheryl acetate" was prepared from natural phytol and thus was 2DL-α-tocopheryl acetate, an equimolar mixture of α-tocopheryl acetate and 2L-α-tocopheryl acetate. The International Standard has often been referred to as *dl*-α-tocopheryl acetate and thus confused with all-*rac*-α-tocopheryl acetate. Use of the new IUNS Nomenclature should eliminate this ambiguity.

In 1962, the Animal Nutrition Research Council introduced the ANRC Vitamin E Reference Standard (5). It is a gelatin-beaded preparation of 2DL-α-tocopheryl acetate and its potency is established on the basis of chemical assay and expressed in International Units. Unfortunately, it is labeled as containing *dl*-α-tocopheryl acetate and this ambiguity should be clarified by specifying that the ANRC Vitamin E Reference Standard contains 2DL-α-tocopheryl acetate synthesized from natural phytol.

VITAMIN E BIOASSAYS

Bioassays for vitamin E can be based on a wide variety of biological responses. They can be categorized depending upon the physiological processes involved as follows: (a) bioassays involving a biological function such as the prevention of fetal resorption in rats or encephalomalacia in chicks; (b) measurement of a physiological parameter, such as prevention of erythrocyte hemolysis; and (c) measurement of vitamin E levels in vivo such as liver storage and plasma responses.

A generalized picture of the physiological processes involved in the various types of vitamin E bioassays is summarized in Figure 2. The rate and the extent of absorption of orally administered vitamin E are controlled by the intestinal membrane barrier mediated by bile salts and other secretions into the intestine. Similarly the absorption of parenterally administered vitamin E is controlled by cellular membrane barriers mediated by the type of emulsifier system in the preparation. Vitamin E is transported both in the lymph and in blood as a protein complex. The extent of tissue storage of vitamin E is determined both by plasma levels and by cellular membrane barriers. Vitamin E presumably exerts its specific biological functions as complexes with intracellular particulate fractions such as mitochondria or microsomes. Levels of vitamin E in these intracellular particulate complexes are mediated not only by cellular membrane barriers but also by specific binding sites.

The specificity of the various types of bioassays for vitamin E in ascertaining the biological value of the tocopherol isomers is determined by the degree of complexity of the physiological processes involved. Vitamin E plasma levels are determined principally by the relative rates of absorption involving a single

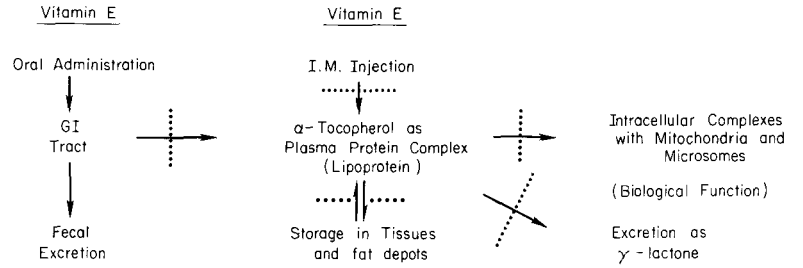

FIG. 2

TABLE I

Biopotency of Alpha-Tocopheryl Acetate[a]

Deficiency symptom and species	Standard (form of tocopheryl acetate)	Number of assays	Biopotency, IU/mg	Reference
Fetal resorption				
Rat	IS	30	1.71[b]	c
Rat	All-rac-α	1	1.36	(9)
Rat	---	1	1.46	(10)
Rat	---	1	1.53	(10)
Encephalomalacia				
Chick	All-rac-α	3	1.68	(11)
Chick	All-rac-α	1	1.36	(12)
Chick	All-rac-α	1	1.77[d]	(12)
Muscle dystrophy				
Chick	ANRC	1	1.53[e]	(14)
Weighted mean biopotency, 39 bioassays[f]			1.675 ± 0.056[g]	

[a]Data from bioassays based on biological function.

[b]Includes data reported earlier by Harris and Ludwig (7) and Ames et al. (8).

[c]S.R. Ames and M.I. Ludwig, unpublished data.

[d]Recalculated from data in their Table VIII fed "in diet" by conventional parallel line bioassay procedures. Calculated ED_{50} values were 0.90 and 1.17 for α and all-*rac*-α-tocopheryl acetate respetively when fed at a weight ratio of 1:1.36. Thus, 1.17/0.90 x 1.36 = 1.77.

[e]Recalculated from data on muscle score by conventional parallel line bioassay procedures. From the log dose-response lines, the calculated ED_Y values were 3.69, 5.65 and 15.6 for α, all-rac-α- and 2L-α-tocopheryl acetate, respectively. Thus, 5.65/3.69 = 1.53. Authors reported ratio of 1.46.

[f]Each separate bioassay reported is given equal weight.

[g]Standard error of the sample mean.

membrane barrier. Tocopherol levels in tissues are determined by somewhat more complex physiological processes involving plasma transport and two membrane barriers. Only those bioassays based on the prevention or reversal of a vitamin E deficiency symptom have the additional specificity involved in the formation of complexes with intracellular particulate fractions. Such bioassays based on a biological function provide the ultimate evaluation of the nutritional value of the various forms of vitamin E.

BIOPOTENCY OF α-TOCOPHERYL ACETATE

α-Tocopheryl acetate (2D,4'D,8'D) is the most active of the common esters of vitamin E. Its biological potency can be determined in two ways: (a) by direct bioassay preferably against the International Standard; however, all-*rac*-α-tocopheryl acetate is often used; and (b) by determining the ratio of the biopotencies of α- and 2L-α-tocopheryl acetates since the International Standard is an equimolar mixture of these two isomers. Data on the biopotency of α-tocopheryl acetate as measured by bioassays based on a biological function of vitamin E are summarized in Table I.

The classical measurement of vitamin E activity is the rat fetal resorption bioassay of Mason and Harris (6). Ames and Ludwig (unpublished data) found that 30 fetal resorption bioassays of α-tocopheryl acetate against the International Standard yielded a mean biopotency of 1.71 IU/mg. Their report includes the one bioassay on α-tocopheryl acetate compared to the International Standard reported earlier by Harris and Ludwig (7) and the three pertinent bioassays reported by Ames et al. (8). Results of fetal resorption bioassays in rats from two other laboratories are included in Table I even though the standards used were not well characterized (9,10).

Dam and Sondergaard (11) have reported that the results of three bioassays based on prevention of encephalomalacia in chicks resulted in an average biopotency of 1.68 IU/mg. Marusich et al. (12) compared the effectiveness of α-tocopheryl acetate and all-*rac*-α-tocopheryl acetate in preventing encephalomalacia. Based on data in Table VII of their paper, they reported a biopotency of 1.36 IU/mg. Data reported in Table VIII of their paper on "in diet" administration were recalculated by conventional "parallel line" bioassay procedures (13) and indicate a potency of 1.77

IU/mg. Using the muscle dystrophy response in chicks, Scott and Desai (14) compared α-tocopheryl acetate with the ANRC Vitamin E Reference Standard. They reported a potency of 1.46. Recalculation of their data on muscle dystrophy scores by conventional "parallel line" bioassay procedures (13) indicated a potency of 1.53 IU/mg. The differences between these three biological function bioassays in two species were not significant. Based on a total of 39 bioassays, the weighted mean biopotency of 1.675 IU/mg is established for α-tocopheryl acetate.

An alternate method of establishing the biopotency of α-tocopheryl acetate is by measuring the ratio of the biopotencies of 2L-α-tocopheryl acetate and α-tocopheryl acetate. As described in detail in a subsequent paragraph, this ratio based on 18 bioassays employing five different biological functions in four species is 0.213. Since the former International Standard is an equimolar mixture of α-tocopheryl acetate and its 2L-epimer and has a defined potency of 1 IU/mg, this relationship may be expressed algebraically as

$$0.5 \times \text{Potency of } \alpha\text{-tocopheryl acetate in IU/mg} + 0.5 \times \text{Potency of } 2L\text{-}\alpha\text{-tocopheryl acetate in IU/mg} = 1.00 \text{ IU/mg} \quad [1]$$

and

$$0.5 \times P + 0.5 \times P \times 0.213 = 1.00$$

where P is the biopotency of α-tocopheryl acetate in IU/mg. Solving this equation indicates that the biopotency of α-tocopheryl acetate is 1.65 IU/mg, a value in excellent agreement with the value determined by direct bioassay against the International Standard.

The mean value for these two independent procedures for determining the biological activity of α-tocopheryl acetate is 1.66 IU/mg. This is the best estimate of the true biopotency of α-tocopheryl acetate based on all available data using bioassays based on biological function.

BIOPOTENCY OF 2L-α-TOCOPHERYL ACETATE

2L-α-Tocopheryl acetate (2L4'D,8'D), the 2 epimer of α-tocopheryl acetate, was isolated in pure form from synthetic 2DL-α-tocopheryl acetate by Robeson and Nelan (2) and was synthesized directly by Mayer et al. (15). Data on the biopotency of 2L-α-tocopheryl acetate by bioassays based on biological function are summarized in Table II.

Using the fetal resorption bioassay, Ames et al. (8) have reported that 12 bioassays gave a mean biopotency ratio of 2L-α- to α-tocopheryl acetate of 0.21. Witting and Horwitt (16) found 2L-α-tocopheryl acetate to be 0.20 as active as α-tocopheryl acetate in preventing creatinuria and growth depression in rats. Fitch and Diehl (17) found 2L-α-tocopheryl acetate to be 0.21 as active as α-tocopheryl acetate in preventing muscular dystrophy in the rabbit. Based on prevention of dystrophy and anemia in monkeys, Fitch et al. (18) found 2L-α-tocopheryl acetate to be 0.14 as active as α-tocopheryl acetate. Dam and Sondergaard (11) reported that 2L-α-tocopheryl acetate was 0.26 as active as α-tocopheryl acetate and 0.44 as active as all-*rac*-α-tocopheryl acetate in preventing encephalomalacia. Scott and Desai (14) compared 2L-α-tocopheryl acetate with α-tocopheryl acetate and the ANRC Vitamin E Reference Standard in the prevention of muscular dystrophy in chicks. Recalculation of their data on muscle dystrophy scores by conventional "parallel line" bioassay procedures confirmed their reported relative activity of 0.36 for 2L-α-tocopheryl acetate compared with 2DL-α-tocopheryl acetate and determined a relative activity of 0.24 for 2L-α-tocopheryl acetate compared with α-tocopheryl acetate. Based on a total of 18 bioassays employing five different biological functions in four species, the mean ratio of 2L-α-tocopheryl acetate to α-tocopheryl acetate was 0.213 and the mean biopotency of 2L-α-tocopheryl acetate is 0.35 IU/mg (compared with 2DL-α-tocopheryl acetate).

RELATIVE ACTIVITIES OF TOCOPHERYL ACETATES BASED ON OTHER BIOLOGICAL TESTS

The observation by Gyorgy and Rose (19) that the red blood cells of vitamin E deficient rats are hemolyzed by dialuric acid was the basis of the hemolysis bioassay for vitamin E as developed by Rose and Gyorgy (20) and modified by Friedman et al. (21) and Bruggemann et al. (22). Supplementation of vitamin E deficient rats with progressively larger doses of vitamin E results in a progressive decline in erythrocyte susceptibility to hemolysis. The response to oral supplements is determined by the extent of intestinal absorption, plasma transport and incorporation into specific binding sites in the erythrocyte membrane. Thus, the hemolysis bioassay is intermediate between bioassays based on biological function and those involving measurement of vitamin E levels in plasma or liver tissue.

Data comparing orally administered α-tocopheryl acetate, 2L-α-tocopheryl acetate and all-*rac*-α-tocopheryl acetate in the erythrocyte

TABLE II

Biopotency of 2L-α-Tocopheryl Acetate[a]

Deficiency symptom and species	Standard (form of tocopheryl acetate)	Number of assays	Relative activity, 2L-α/α	Biopotency[b] IU/mg	Reference
Fetal resorption, rat	α-	12	0.21	(0.35)	8
Creatinuria-growth rat	α-	1	0.20	(0.33)	16
Muscle dystrophy, rabbit	α-	1	0.21	(0.35)	17
Dystrophy-anemia, monkey	α-	1	0.14	(0.24)	18
Encephalomalacia, chick	All-rac-α-	2	0.26	0.44	11
Dystrophy, chick	ANRC	1	0.24[c]	0.36[d]	14
Weighted mean, 18 bioassays[e]			0.213 ± 0.007[f]	0.353 ± 0.012[f]	

[a]Data from bioassays based on biological function.

[b]Values in parentheses are calculated algebraically using Equation 1.

[c]Recalculated from data on muscle score as described in Footnote e, Table I. Thus, 3.69/15.6 = 0.24 essentially confirming authors' reported value of "approximately 25%".

[d]Recalculated from data on muscle score as described in Footnote e, Table I. Thus, 5.65/15.6 = 0.36 confirming authors' reported value of 0.36.

[e]Each separate bioassay is given equal weight.

[f]Standard error of the sample mean.

hemolysis bioassay are summarized in Table III. Friedman et al. (21) reported that 10 bioassays of α-tocopheryl acetate compared to all-*rac*-α-tocopheryl acetate gave a mean ratio of 1.47. Weiser et al. (23) stated that their results were in agreement with a 1.36 ratio but recalculation of their reported data following arc sine transformation (13) yielded a ratio of 1.61 for α-tocopheryl acetate compared with all-*rac*-α-tocopheryl acetate. Gloor (10) was reported to have found a ratio of 1.44 for a similar comparison. Bruggemann et al. (22) reported on hemolysis bioassays of several different preparations of α-tocopheryl acetate and comparisons with samples of 2DL-α-tocopheryl acetate. These are separately tabulated and gave mean ratios of 1.74, 1.66, 2.05 and 1.05. Ames and Ludwig (unpublished data) compared α-tocopheryl acetate four times against the International Standard in the hemolysis bioassay in rats and found a mean ratio of 1.47. The ratio of 1.55 recalculated from hemolysis tests in humans (24) will be discussed later. These 28 bioassays yield a weighted mean ratio for α-tocopheryl acetate with either all-*rac*-α-tocopheryl acetate or the International Standard of 1.53 for the hemolysis bioassay.

A limited number of hemolysis bioassays comparing 2L-α-tocopheryl acetate with either α-, all-*rac*- or 2DL-α-tocopheryl acetates are also summarized in Table III. The calculated mean ratio of 2L-α-tocopheryl acetate compared with all-*rac* or 2DL-α-tocopheryl acetate of 0.56 is significantly greater than the comparable ratio of 0.35 determined by bioassays based on biological function.

Responses of α-tocopheryl acetate in other biological tests are summarized in Table IV. Dunkley et al. (25) compared α-tocopheryl acetate and all-*rac*-α-tocopheryl acetate in the dairy cow. The relative efficiency of secretion of α-tocopherol in milk following oral supplementation of the acetate esters was 1.43.

Two chick liver storage bioassays for vitamin E have been described by Pudelkiewicz et al. (26) and by Dicks and Matterson (27) differing principally in the length of time the supplements were fed. A mean ratio of 1.34 for α- compared with all-*rac*-α-tocopheryl acetate was found in five tests using a supplementation period of 14 days (26). A mean ratio of 1.17 was found for three 14-day bioassays and a mean ratio of 1.30 for three 3-day bioassays involving a 3-day supplementation period (27). Subsequently, Matterson (5) summarized the chick liver storage determinations in his labora-

TABLE III

Responses of Tocopheryl Acetates in the Erythrocyte Hemolysis Bioassay

Responses of α-tocopheryl acetate					Responses of 2L-α-tocopheryl acetate					
Species	Standard (form of tocopheryl acetate)	Number of tests	Relative activity per mg α/standard	Reference	Species	Standard (form of tocopheryl acetate)	Number of tests	Relative activity, 2L-α/α	Relative activity, 2L-α/all-rac-α or 2DL-α	Reference
Rat	All-rac-α	10	1.47	21	Rat	All-rac	1	---	0.625[c]	23
Rat	All-rac-α	1	1.61[a]	23	Rat	α-	1	0.44	(0.61)[e]	23
Rat	---	1	1.44	10	Rat	α-	1	0.415[d]	(0.51)[e]	23
Rat	2DL	4	1.74	22	Rat	2DL-α	1	---	0.51	22
Rat	2DL	4	1.66	22	Rat	α-	3	0.35	(0.52)[e]	f
Rat	2DL	1	2.05	22						
Rat	2DL	2	1.05	22						
Rat	2DL	4	1.47	f						
Human	All-rac-α	1	1.55[b]	24						
Weighted mean relative activity		28	1.533 ± 0.048[g]						0.556 ± 0.022[g]	

[a]Recalculated from authors' data by conventional bioassay procedures following arc sine transformation of the sigmoid log dose-response curves. Calculated ED_{50} values were 0.852, 1.369 and 2.092 for alpha-, all-rac-α-, and 2L-α-tocopheryl acetate, respectively. (Corresponding ED_{50} values of 0.845, 1.374 and 2.096 following probit transformation are essentially the same). Based on ED_{50} values following the arc sine transformation as used by the authors, the relative activity of alpha- to all-rac-α-tocopheryl acetate is 1.369/0.852 = 1.61.

[b]Recalculated from authors' data by conventional bioassay procedures following probit transformation of the sigmoid log dose-response curves. Since the responses for the two highest dose levels gave essentially zero hemolysis, those for the highest dose level were omitted. ED_{50} values were 11.45 and 17.72 for alpha- and all-rac-α-tocopheryl acetate, respectively. Thus, 17.72/11.45 = 1.55.

[c]Authors reported value. Recalculation from authors' data as described in footnote b, Table II (1.369/2.092 = 0.655) essentially confirmed this value.

[d]Authors reported value. Recalculation from authors' data as described in footnote b, Table II (0.852/2.092 = 0.407) essentially confirmed this value.

[e]Values in parentheses are calculated algebraically from equation 1.

[f]S.R. Ames and M.I. Ludwig, unpublished data.

[g]Standard error of the sample mean.

TABLE IV

Responses of α-Tocopheryl Acetate in Other Biological Tests[a]

Response, species	Standard (form of tocopheryl acetate)	Number of tests	Relative activity per mg. α/standard	Reference
Secretion in milk,				
cow	All-rac-α	1	1.43	25
Liver storage,				
Chick	All-rac-α	5	1.34[b]	26
Chick	All-rac-α	3	1.17[b]	27
Chick	All-rac-α	3	1.30[c]	27
Chick	All-rac-α	---	1.34[b]	5
Chick	All-rac-α	---	1.21[c]	5
Rat	All-rac-α	1	2.04[c]	28
Plasma levels,				
Chick	All-rac-α	5	1.36	26
Chick	All-rac-α	3	1.36	12
Chick	ANRC	1	1.17[d]	14
Chick rat, calf	All-rac-α	9	1.36	29
Human	All-rac-α	1	3.48[e]	24
Mean relative activity		33	1.346[f]	

[a]Data from assays not based on biological function.

[b]Fourteen-day liver storage bioassay.

[c]Three-day liver storage bioassay.

[d]Recalculated from the authors' data using conventional slope ratio bioassay procedures. Slopes of the regression lines were 77.37 and 65.93 for α- and all-rac-α-tocopheryl acetate, respectively. Thus, 77.37/65.93 = 1.17.

[e]Recalculated from the authors' data using conventional parallel line bioassay procedures. Plasma tocopherol levels following supplementation were corrected by subtracting corresponding presupplementation plasma levels. ED_Y values were 13.68 and 47.57 for α- and all-rac-α-tocopheryl acetate, respectively. Thus, 47.57/13.68 = 3.48.

[f]Omitting value of 3.48 resulting from recalculation of Horwitt's data.

tory and reported ratios of 1.34 and 1.21 for α- compared with all-*rac*-α-tocopheryl acetate for the 14-day and 3-day bioassays, respectively. However, the number of bioassays of the acetate ester was not specified. Quaife (28) has reported that preliminary data based on three-day liver storage tests in rats indicated a ratio of 2.04 for α- compared with all-*rac*-α-tocopheryl acetate.

Elevation of plasma tocopherol levels has been used by several investigators to estimate vitamin E responses. This type of biological test has the least specificity since it is dependent principally on the rate of absorption and does not involve measurement of a biological function. Furthermore, the tocopherols found by chemical assay may not have the same biological value. Determination of the ratio of biological response of α- to all-*rac*-α-tocopheryl acetate in the chick has been reported as 1.36 (five tests) by Pudelkiewicz et al. (26) and as 1.17 (one recalculated slope ratio bioassay) from the data of Scott and Desai (14). Marusich et al. (29) have reported that in a series of single tests involving administration orally in the diet, orally as a single dose, and by intramuscular injection in rats, chicks and calves, the ratio of response of α- to all-*rac*-α-tocopheryl acetate was about 1.36 (nine tests in all). Some of the plasma responses in chicks reported earlier (12) appear to be included in the data referred to above.

Data in humans comparing the relative responses of α- and all-*rac*-α-tocopheryl acetate are limited to that reported by Horwitt (24). He maintained adult humans on a vitamin E deficient diet until plasma tocopherol levels were low and then measured plasma tocopherol and erythrocyte hemolysis at intervals during a 138-day repletion period. Supplements of all-*rac*-α-tocopheryl acetate were fed at 1.33 times as much by weight as the level of corresponding supplements of α-tocopheryl acetate. Substantially greater responses were obtained for α-tocopheryl acetate than for all-*rac*-α-tocopheryl acetate. The hemolysis responses

were recalculated using a probit transformation and indicated a ratio of activities of α- to all-*rac*-α-tocopheryl acetate of 1.55. Horwitt's data on the plasma tocopherol responses corrected for zero time were recalculated as a parallel line bioassay and indicated a ratio of activities of α- to all-*rac*-α-tocopheryl acetate of 3.48.

BIOLOGICAL ACTIVITY OF OTHER DIASTEREOISOMERS

Only two of the eight possible diastereoisomers of α-tocopheryl acetate have been isolated in pure form and their biopotencies determined. These two isomers, α- and 2L-α-tocopheryl acetate have a fivefold difference in biopotency indicating the importance of the configuration at C-2 in determining biological activity. Ames et al. (8), noting the similarity of the biopotencies of 2DL- and all-*rac*-α-tocopheryl acetates in the fetal resorption bioassay, concluded that the configuration at C-2 was apparently dominant in determining biological activity. However, this conclusion was based on only five bioassays; three bioassays of two preparations of 2DL-α-tocopheryl acetate and one bioassay of each of two preparations of all-*rac*-α-tocopheryl acetate. Friedman et al. (21) stated that a sample of all-*rac*-α-tocopheryl acetate assaying 93% pure by gold chloride titration was 94% as active as the International Standard in the hemolysis bioassay (weighted average of four comparisons). On the other hand, Bruggemann et al. (22) reported that a sample of all-*rac*-α-tocopheryl acetate was only 85% as active as 2DL-α-tocopheryl acetate based on two hemolysis bioassays. In view of the limited data, the identity of the biopotencies of 2DL-α-tocopheryl acetate and all-*rac*-α-tocopheryl acetate has not been adequately demonstrated.

A carbon-labeled preparation of the four 2D-isomers of α-tocopheryl acetate (2R,4'RS,8'RS-α-tocopheryl acetate and inappropriately named "*d*"-α-tocopheryl acetate) and a tritium-labeled preparation of the four 2L-isomers of α-tocopheryl acetate (2S,4'RS,8'RS-α-tocopheryl acetate and inappropriately named "*l*"-α-tocopheryl acetate) were synthesized by Mayer et al. (15). Their detailed compositions were stated to be as follows:

2D,4'D,8'D 2D,4'D,8'L 2D,4'L,8'D 2D,4'L,8'L	"*d*"-α-tocopheryl acetate (2D-isomer mixture)
2L,4'D,8'D 2L,4'D,8'L 2L,4'L,8'D 2L,4'L,8'D	"*l*"-α-tocopheryl acetate (2L-isomer mixture)

These two preparations were fed separately and in equimolar mixture to two rats by Weber et al. (30), and levels of radioactivity in blood, liver and heart muscle determined. The equimolar mixture presumably had the same composition as all-*rac*-α-tocopheryl acetate (an equimolar mixture of the eight possible isomers). From their graphical data on levels of total radioactivity in tissues, biological activity ratios for the 2D-isomer mixture compared with all-*rac*-α-tocopheryl acetate were recalculated by assuming that all radioactivity is as tocopherol isomers. The ratios based on blood levels were 0.91 and 1.02 at 2 and 96 hr, respectively, and the corresponding ratios based on liver levels were 0.63 and 0.89. These activity ratios range from 47% to 75% of the mean ratio of 1.35 for α- and all-*rac*-α-tocopheryl acetate (Table IV) based on many liver storage and plasma level determinations. These data indicate that one or more of the 2D-isomers in the mixture has significantly less activity than α-tocopheryl acetate (2D,4'D,8'D)and that the configurations at C-4' and C-8' apparently can significantly affect the biological activity of diastereoisomers of α-tocopheryl acetate. Furthermore, these data suggest that the biological activities of the four enantiomeric pairs of diastereoisomers may not be the same as inferred by Ames et al. (8). Resolution of the problem of the biopotencies of the other six diastereoisomers of α-tocopheryl acetate must await their preparation and bioassay.

ABSORPTION AND EXCRETION OF THE DIASTEREOISOMERS OF α-TOCOPHERYL ACETATE

The relative rates of absorption of the corresponding tocopherols and excretion of the 2D- and 2L-isomer mixtures described above were investigated by Weber et al. (31) using orally administered tritium-labeled preparations. The 2L-isomer mixture was absorbed more rapidly but excreted more rapidly than the 2D-isomer mixture. One half hour after administration, there was more radioactivity from the 2L-isomer mixture than from the 2D-isomer mixture in all tissues examined, but at 2 hr, the pattern was reversed. Twenty-four hours after administration, almost twice as much radioactivity from the 2D-isomer mixture as from the 2L-isomer mixture was retained in depot fat and 92% as much in liver. Both preparations were metabolized to tocopherol quinone (32) and excreted as the γ-lactone metabolite (33). However, three times as much of the metabolite was excreted in 24 hr following administration of the 2L-isomer mixture than with the 2D-isomer mixture.

Desai et al. (34) investigated the absorption as the corresponding tocopherols of tritium-labeled preparations of α-tocopheryl acetate and 2L-α-tocopheryl acetate as Tween emulsions from ligated intestinal loops of chicks. 2L-α-Tocopheryl acetate was more rapidly absorbed from duodenal loops but not from jejunal or ileal loops. Desai and Scott (35) observed that the retention of 2L-α-tocopherol in chick plasma was about 25% that of α-tocopherol 48 hr after dosing with the corresponding acetate esters, and that selenium supplementation lowered this relative retention. Scott (36) has proposed that a biological role of selenium may lie in a selenium-containing compound which acts as a carrier of vitamin E in physiological processes. Thus data on both rats and chicks indicate that the 2L-isomeric α-tocopherols fed as the acetate esters are more rapidly absorbed and much more rapidly excreted than the 2D-isomeric forms.

These differences in rates of absorption and excretion explain why the relative activities of α- and 2L-α-tocopheryl acetate are different in the three categories of biological responses. Since the 2L-isomer is excreted more rapidly than the 2D-isomer, plasma responses can vary widely depending on the time following the administration of the last dose. The more rapid excretion of 2L-α-tocopherol is also a rational explanation for the different relative activities found by Matterson (5) for the 14-day and 3-day liver storage bioassays in chicks. The longer feeding period would result in less storage of 2L-α-tocopherol from the standard, 2DL-α-tocopheryl acetate, and a higher relative value for the activity ratio of α-tocopheryl acetate to the standard. The liver storage bioassay is valid for measuring the potencies of different preparations of α-tocopheryl acetate but not for comparison of the relative activities of different isomers of vitamin E. These observations emphasize the necessity of relying only on data from bioassays based on biological function, to ascertain the biopotencies of the different tocopherol isomers.

POSSIBLE SYNERGISM

The absorption and tissue deposition of a carbon-labeled mixture of 2D-isomeric α-tocopheryl acetates and a tritium-labeled mixture of 2L-isomeric α-tocopheryl acetates when fed separately and in an equimolar mixture (30) were referred to above. Levels of radioactivity in blood, liver and heart muscle following administration to two rats of the equimolar mixture were about 55% higher than expected from data on the separate isomer mixtures. These observations suggested a synergistic effect of the two isomer mixtures and the authors considered that the calculation of the biopotency of an isomer mixture from that of the single components may not be justified. As was noted above, the activity ratios calculated from the data of Weber et al. (30) for the 2D-isomer mixture and all-*rac*-α-tocopheryl acetate were not compatible with the ratios determined for α-tocopheryl acetate and all-*rac*-α-tocopheryl acetate (Table IV).

A series of mixtures of α- and 2L-α-tocopheryl acetate were compared using the fetal resorption bioassay in rats by Ames et al. (8). The regression line of per cent 2L-isomer composition on biological response was linear from 0% to 100%. Synergism between these two isomers would have resulted in a curvilinear regression line with higher responses especially at the point of 50% L-isomer composition but this was not observed. These data show that there is no synergistic effect between α-tocopheryl acetate and 2L-α-tocopheryl acetate as exemplified by the equimolar mixture comprising the former International Standard. Scott and Desai (14) found that the response for α-tocopheryl acetate in the prevention of muscular dystrophy in chicks was 12% (recalculated value is 8%) lower than predicted and ascribed this difference to synergism. However, observation of an 8% or 12% lower value in a single bioassay can be ascribed easily to normal biological variation. Witting and Horwitt (16) found no evidence of synergism or sparing action between α- and 2L-α-tocopheryl acetate in tests in rats involving prevention of creatinuria and growth depression.

A critical evaluation of possible synergism between α- and 2L-α-tocopheryl acetate can be made from all the bioassay data involving biological function. Substitution of the mean weighted biopotencies determined from the data of Tables I and II in Equation 1 yields

$$0.5 \times 1.675 + 0.5 \times 0.35 = 1.01 \qquad [2]$$

Which is in excellent agreement with the defined biopotency of the International Standard, 2DL-α-tocopheryl acetate, or 1.00 IU/mg. If a mixture of α- and 2L-α-tocopheryl acetate exhibited synergism, then the enhanced activity of the 2DL-α-tocopheryl acetate would result in depressed ratios of α- to 2DL-α-tocopheryl acetate and 2L- to 2DL-α-tocopheryl acetate. Summation of the respective biopotencies as in Equation 2 would result in a value significantly less than unity if synergism were present. A similar summation for the responses of the separate isomers in the hemolysis bioassay (Table III) indicates that

$$0.5 \times 1.533 + 0.5 \times 0.556 = 1.04 \qquad [3]$$

again not significantly different from unity and indicating no synergism.

Thus, the evidence from all bioassays based on biological function as well as on the erythrocyte hemolysis test confirm the conclusion based on data from direct bioassay. Mistures of α-tocopheryl acetate and its 2L-epimer do not exhibit synergism and enhanced biological activity.

NEED FOR A NEW INTERNATIONAL STANDARD FOR VITAMIN E

Many of the problems associated with the evaluation of the biopotencies of the isomers oof α-tocopheryl acetate could be resolved by authorization of a new International Standard for vitamin E and by redefining the International Unit in terms of the new International Standard. The former International Standard is no longer available. 2DL-α-Tocopheryl acetate can be synthesized from natural phytol but any purification of the reaction mixture may change, and crystallization will change, the isomer ratio. Since 2DL-α-tocopheryl acetate is by definition an equimolar mixture of two isomers, it is difficult to prepare it in the purity normally required for a reference standard. Furthermore, only half of this mixture of two of the eight possible diastereoisomers of α-tocopheryl acetate corresponds to the naturally occurring and biologically most active form. These two isomers are absorbed, stored in tissues, metabolized and excreted at different rates. Difficulties in its use as a bioassay standard are obvious.

The use of α-tocopheryl acetate as a new International Standard would have many advantages. α-Tocopheryl acetate is readily available in high purity. Since it is a single isomer, its isomeric composition cannot be altered by crystallization or other purification procedures. It is the isomer corresponding to naturally occurring vitamin E, thus facilitating interpretation of the vitamin E potencies of foods and feeds. As a single pure isomer of vitamin E, it would have replicable absorbability, tissue storage, metabolism and excretion thus rendering possible valid interlaboratory comparisons.

Similar advantages would be associated with the redefinition of the International Unit. It is illogical to define the International Unit in terms of a mixture of isomers of different biological activities that furthermore is unavailable. By defining one International Unit as equal to 0.60 mg of pure α-tocopheryl acetate, the new Unit would be closely equivalent to that of 1.0 mg of the former International Standard. The appropriate committees of international organizations are urged to consider these recommendations.

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Tocopherols in Foods and Fats[1]

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ABSTRACT

Methods are now available for the determination of all the specific tocopherol forms found in nature. Although the greatest interest centers on alpha-tocopherol, much information has been gathered on the amounts of individual tocopherols in foods and fats contributing to the human diet. This paper summarizes and discusses the recent literature on the tocopherols in natural, processed and prepared foods. Alpha-tocopherol, although the most widely distributed, is in many instances not the predominant form. In a number of important tocopherol sources, e.g., soybean oil, much larger amounts of gamma-tocopherol are found. The levels of tocopherols are variable, but the evidence suggests that the identities of the specific forms are characteristic of the source. In cereal grains the further observation may be made that the related tocol-tocotrienol pairs tend to be found together. Processing and preparation almost invariably reduce the tocopherol content, sometimes to insignificant levels.

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INTRODUCTION

In the half century since the discovery of vitamin E much has been published on the amounts of this nutrient in various foods and fats. Most of this literature has given data for the total tocopherol content, rather than for the individual forms. Only recently have the tocopherols that occur in nature been fully identified and methods developed for their determination. These methods, using paper, thin layer (TLC) and gas chromatography (GC), have been applied to many foods and fats, but our knowledge of both the amounts and the forms to be expected in various foods is far from complete. Harris (1), in a review published in 1962 on the retention of vitamin E in stored and processed foods, concluded that there was at that time essentially no published data on the individual tocopherols in foods. Dicks (2) compiled all of the information on the vitamin E content of foods and feeds available up to 1965: only about 5% of the entries gave values for the individual forms. This paper reviews the recent literature on the tocopherols in foods, fats and their plant sources, concentrating on that appearing since 1964, and attempts to assess our current knowledge of the identities and amounts to be expected.

The naturally occurring forms are eight in number: alpha-, beta-, gamma- and delta-tocopherol (α-T, β-T, γ-T and δ-T), and alpha-, beta-, gamma- and delta-tocotrienol (α-T-3,

TABLE I

Tocopherols in Seeds, mg/100 g

Seed	Number of values averaged	α-T	α-T-3	β-T	β-T-3	γ-T	γ-T-3	δ-T	References
Almond	2	27.4	0.5[a]	0.3[a]		0.9[a]			5[b]
Barley	5	0.4	1.3	0.3	0.7[e]	0.05[d]	0.2[c]	0.01[a]	5-9
Corn	11	0.6	0.2[f]		0.4[a]	3.8	0.5[f]	Trace	5,8-11[b]
Millet	1	0.05		Trace		1.3		0.4	5
Oats	6	0.7	0.7	0.2[d]	0.1[d]	0.3[d]			5-7,9-11
Peanuts	1	9.7				6.6			[b]
Peas	3	0.5				6.4[c]		0.6[a]	8,12
Pecans	1	1.2				19.1			[b]
Poppyseed	1	1.8				9.2			5
Rice	5	0.3	Trace			0.3[d]	0.5[c]	0.04[e]	11,12[b]
Rye	4	0.8	1.3[d]	0.4[d]	0.9	0.6[a]			5-7,9
Walnut, English	1	0.4				15.8		1.3	[b]
Wheat	9	1.0	0.4[h]	0.9	2.5[g]			0.08[a]	5,7-11,13

[a]One value reported.
[b]Slover, unpublished data.
[c]Average of two values.
[d]Average of three values.
[e]Average of four values.
[f]Average of six values.
[g]Average of seven values.
[h]Average of eight values.

TABLE II

Tocopherols in Grains, Determined by Gas Liquid Chromatography and Identified by Retention on Three Stationary Phases, mg/100 g, Fresh Weight[a]

Grain	α-T	α-T-3	β-3	β-T-3	γ-T	γ-T-3	δ-T
Barley	0.2	1.1	0.4	0.3	0.03	0.2	0.1
Corn, fresh, yellow	0.06	0.2			0.4	0.4	
Corn, dry, yellow	0.6	0.3			4.5	0.5	
Millet	0.05		Trace		1.3		0.4
Oat	0.5	1.1	0.1	0.2			
Rice, white	0.1	Trace			0.1	0.2	Trace
Wheat	1.4	0.5	0.7	3.3			

[a]Reference 5,11 and Slover, unpublished data.

β-T-3, γ-T-3 and δ-T-3) (3). All these forms have been found in foods and fats, although δ-T-3 is rare and, among foods, has been reported only in palm oil.

Seeds and Seed Oils

All natural tocopherols are derived from plant sources in which they are concentrated principally in the seeds. Although most analytical procedures do not distinguish the free and esterified forms, it is commonly assumed that tocopherols occur mainly as the free alcohol. Chow et al. (4) have determined both free and esterified tocopherols in a number of food oils, using a method not requiring saponification. Only the free alcohols were found in oils from barley, corn, oats, soybeans and wheat bran, indicating the absence of any significant amount of tocopherol esters.

Published data on the forms in seeds have varied both with regard to kind and amount. The values given in Table I are averages of the amounts reported for each tocopherol. Of the eight naturally occurring forms only δ-T-3 is missing. α-T has been found in all seeds but was the major form only in three; almonds, oats and peanuts. Other than this there are a few other instances of general agreement. There is a consensus that barley contains α-T, α-T-3 and β-T; corn α-T and γ-T; oats α-T and α-T-3; and rye α-T and β-T-3. Although some authors have not reported β-T or β-T-3 in wheat grain, there is ample evidence that these forms occur in addition to α-T and α-T-3. Data are available for only a few nuts; these contain principally γ-T and α-T. Almonds were the richest in α-T, averaging 27.4 mg/100 g.

In our laboratory we have determined the tocopherols in samples of most of these seeds by GC, basing each identity on relative retentions on three gas chromatographic stationary phases (Table II). The tocopherol patterns were distinguished by the number of forms and by the relatively high levels of toco-

TABLE III

Tocopherols in Vegetable Oils, mg/100 g

Oil	Number of values averaged	α-T	α-T-3	β-T	β-T-3	γ-T	γ-T-3	δ-T	δ-T-3	References
Coconut	1	0.5	0.5		0.1		1.9	0.6		5
Corn	8	11.2		5.0[a]		60.2		1.8[b]		4,5,15-18
Cottonseed	9	38.9				38.7				5,16,14-22
Neem	1					58		59		20
Olive	4	5.1								5,21
Palm	4	25.6	14.3[a]		3.2[a]	31.6[b]	28.6[a]	7.0[a]	6.9[a]	10,15,19-22
Peanut	11	13.0				21.4		2.1[c]		5,16-23
Rapeseed	5	18.4				38.0		1.2[a]		15,16,22,24
Safflower	3	38.7				17.4		24[a]		5,19,20
Sesame	2	13.6				29.0				10,20
Soybean	14	10.1				59.3		26.4[d]		5,10,15-22,24,26
Sunflower	10	48.7				5.1		0.8[b]		17-19,21,22,26,27
Walnut	1	56.3				59.5		45.0		25
Wheat germ	3	133.0	2.6[a]	71.0	18.1[b]	26.0[a]		27.1[a]		5,16,17
Mustardseed	1	8.6				17.6		5.8		16

[a]One value reported.
[b]Average of two reported values.
[c]Average of four reported values.
[d]Average of 12 reported values.

TABLE IV

Tocopherols in Peanut Oils, mg/100 g[a] (22)

Genotype	Number of individual varieties	α-T	γ-T	δ-T
Spanish	4	8.4-13.0 (10.7)[b]	20.4-23.8 (22.6)	0.8-2.2 (1.3)
Virginia	7	13.7-19.7 (16.4)	19.5-28.8 (22.0)	0.8-1.4 (1.1)
Runner	6	12.2-23.0 (16.6)	20.0-30.6 (26.6)	0.7-2.5 (1.5)

[a]Average values in parentheses.

trienols. In corn, oats, rye and wheat the related saturated-unsaturated pairs were found together; barley and rice followed the same pattern except for the δ-T/δ-T-3 pair, from which δ-T-3 was missing. γ-T-3 was found in both fresh and dry corn, and was a major form in the fresh corn. Its presence in rice is to be expected, since it was first found in Japanese rice by Green and Marcinkiewicz (13), although then identified as 7-methyltocol.

Vegetable oils, in contrast to seeds, generally have simpler tocopherol patterns and contain principally the saturated forms α-T, γ-T, and δ-T (Table III). β-T in wheat germ oil is to be expected, but its presence in corn oil rests on one report and requires confirmation. None has been found in corn grain. Some of the reports of δ-T are also doubtful, although its presence in such important oils as soybean oil and peanut oil is well established. In only a few oils is α-T the major form; in most, γ-T predominates. Coconut oil and palm oil have similar patterns, although the amounts in coconut oil are negligible. Although γ-T has been reported in palm oil, Whittle and Pennock (14), using two-dimensional TLC, found equivalent amounts of γ-T-3 instead. On the basis of these averaged values α-T accounts for about one third of the total tocopherol in the oils.

It is to be expected that botanical varieties or genotypes of the same plant source would have different tocopherol contents, although this has not been extensively investigated. Stürm et al (22) have determined α-T, γ-T and δ-T in peanut oils from seed from 17 individual varieties of three genotypes of peanuts, Spanish, Virginia and Runner, grown under the same conditions and selected for soundness and maturity (Table IV). There were differences both among genotypes and among varieties of the same genotype: α-T was lowest in the Spanish varieties. The richest sources for all three tocopherol forms were found among the Runner varieties.

These genetic effects may often be difficult to distinguish from those due to differences in maturity and environmental temperature. It was found many years ago by Green (7) that the pattern of tocopherols in some seeds changes greatly during maturation so that differences in maturity at harvest may affect the tocopherol content. Environmental temperature has also been found to affect not only the tocopherol content but also the unsaturation of the fatty acids in some seed oils. Beringer and Saxena (27) carried out pot experiments with oats, sunflower and flax held at either 12 C or 28 C during seed development, and determined α-T + α-T-3 and the fatty acid composition of the extracted oil. The effect on oats was minor, but sunflower seed oil from seeds grown at 28 C contained more tocopherol (68 vs. 28 mg/100 g) and less linoleate (30% vs. 67%) than those grown at 12 C. These findings suggest that seed from different growing areas or grown in different seasons may vary in tocopherol content. However, Piorr et al. (28) have determined α-T and γ-T in sunflower seed oils from three varieties grown in South Africa, Hungary or the USSR in 1965 and 1966 and found no major differences among them. The effect of the three variables, variety, growing area and crop year, was small. In 16 of 17 samples α-T fell between 59 and 73 mg/100 g oil. γ-T was much lower and more variable (1.9-8.3 mg/100 g). The effects of the growing environment on the tocopherol content of this and other seed oils needs further study.

Other factors such as storage, processing and use have also been examined. Gracian and Arevalo (20) determined α-T, γ-T and δ-T in a number of fresh, stored and refined food oils. Fresh virgin olive oil contained only α-T at 15.5 mg/100 g oil; stored virgin olive oil contained only 4.1 mg/100 g; while the refined oil was devoid of all tocopherol. The differences between crude and refined soybean oil were not so clear cut; the crude oil contained 11.5, 35.7 and 27.0 mg of α-T, γ-T and δ-T per 100 g oil; refined oils contained 16.0 51.5 and 26.9

TABLE V

Tocopherols in Vegetables and Fruits, mg/100 g

Vegetable and fruit	α-T	α-T-3	β-T	β-T-3	γ-T	δ-T	References
Asparagus (fresh weight	1.8		0.05		0.07		Slover, unpublished data
Carrots (fresh weight)	0.51	0.04	0.01	0.08			Slover, unpublished data
Cucumber	8.4						12
Mango, flesh, green	0.26					0.27	12
Mango, flesh, ripe	0.98						12
Muskmelon	10.1						12
Tomato	18.2						12

mg/100 g. This merely indicates that although processing may be expected to decrease the tocopherol content, crude oils will not always contain more than refined oils because of their natural variation.

Vegetables and Fruits

The amounts of tocopherols found in vegetables and fruits are small compared to those in seeds and seed oils. A few values are given in Table V, principally from the work of Mannan and Ahmed (11) who analyzed a number of foods grown in East Pakistan. The values of α-T for cucumber, muskmelon and tomato seem high, considering the amount of water in these vegetables. Booth and Bradford reported much lower values for these commodities in their extensive survey of α-T in fruits and vegetables (29). In a discussion of the amount of α-T in vegetables Booth (30) has suggested that in general dark slow-growing leaves are richest, containing 10-40 mg/100 g fresh weight, that stems and leaves contain 1.0-10 mg/100 g, fruits, 0.5-20 mg/100 g, and that the content of α-T increases with maturity. He has also pointed out that leaves contain an enzyme that destroys tocopherols when the leaf is damaged, creating a problem in both food preparation and analysis. The enzyme requires oxygen and may be deactivated by heating. Cooking in boiling water for 30 min destroyed the enzyme and caused a loss of only 8% or less of α-T in brussels sprouts, cabbage and carrots (31). The activity of this enzyme may account for the low values reported for some vegetables, such as that for asparagus in Table V.

TABLE VI

Tocopherols in Hard, Soft and Durum Wheats, mg/100 g (12)

Wheat	Number of samples	α-T	α-T-3	β-T	β-T-3
Hard	5	1.35	0.47	0.73	3.28
Soft	4	1.24	0.50	0.65	3.04
Durum	2	0.99	0.67	0.48	3.67

Processed and Prepared Foods

Wheat, wheat fractions and wheat baked products have been analyzed more extensively than any other single food. Frazer and Lines (32) studied the changes in flour tocopherols caused by ageing and by treatment of the flour with chlorine dioxide, using two-dimensional paper chromatography to determine α-T, α-T-3 + β-T, and β-T-3. Freshly milled untreated flour contained 0.26, 0.26 and 1.00 mg/100 g, respectively, of these forms. After ClO_2 treatment 17% of the α-T, 34% of the α-T-3 + β-T and 50% of the β-T-3 remained. These results are similar to those of Mason and Jones (8) who found retentions of 14%, 35% and 57% for α-T, β-T, and β-T-3 after ClO_2 treatment. On the other hand Moore et al. (33) found retentions of only 6%, 9% and 16% for α-T, α-T-3 + β-T and β-T-3. Untreated flour stored in an open tin for 190 days retained 35%, 36% and 37% of α-T, α-T-3 + β-T-3 and β-T-3, respectively (32).

As a part of a larger study of wheat nutrients Slover et al. (12) analyzed tocopherols in wheat grains, their flours and products made from them, as well as the forms in a series of consumer wheat products collected in 10 specific locations in the Unted States. Only small amounts of tocopherol survived flour processing and baking; this was especially true of the cake flours which were treated with benzoyl peroxide. There were slight but consistent differences among the hard, soft and durum wheats (Table VI); durum wheat contained the least amounts of the saturated α-T and β-T, and the greatest amounts of the tocotrienols α-T-3 and β-T-3. The tocopherols in baked products were highly variable and depended on the shortening used. The α-T content of whole wheat bread, for example, varied from 0.02 to 0.46 mg/100 g (dry weight), while γ-T varied from traces to 1.40 mg; β-T, β-T-3

and δ-T were also found in all samples. Tocopherol patterns varied from that characteristic of lard to that expected from soybean oil-based shortening.

Bunnel et al. (34) have analyzed a number of typical American foods, as eaten, for their α-T and total tocopherol content, and calculated the α-T in typical American breakfast, lunch and dinner menus. These menus provided a daily intake of α-T ranging from 2.6 to 15.4 mg, with an average of 7.4. Many of the higher values included substantial contributions from items such as margarine and pastry whose tocopherol content is derived from vegetable fat. They found that frozen foods fried in vegetable oils were lower in tocopherol than expected, suggesting extensive destruction during freezer storage.

Herting and Drury (10), in work on the tocopherols in corn, wheat, oats and rice focussed their attention on the α-T content of these grains and products made from them. For some products they also reported values for the γ-T in corn and rice, β-T and α-T-3 in wheat, and γ-T and α-T-3 in oats. The amounts of α-T in whole corn, wheat, oats and rice averaged 1.53, 0.87, 1.54 and 0.35 mg/100 g, respectively. Up to 90% of the α-T was lost in processing.

Dairy Products and Infant Foods

Herting and Drury have also reported values for the tocopherols in bovine and simulated milks (9). Two samples of simulated milk contained α-T at 5.8 and 21.7 mg/100 g lipid and γ-T at 27.8 and 35.8 mg/100 g lipid. In contrast, cow's milk contained only α-T at 1.7 mg/100 g lipid. They also found in further work on milk and milk substitutes (35) that simulated milks were also richer than human milk, which contained an average of 1.14 mg α-T/qt. Infant formulas based on vegetable oils, however, contained 2.30-7.67 mg α-T/qt, plus up to 15.2 mg γ-T and 4.8 mg γ-T. Dicks-Bushell and Davis (36) have also investigated the tocopherol content of infant foods. They determined the amounts in 5 infant formulas and 10 infant cereals. The α-T in formulas varied from 0.08 to 1.06 mg/100 g; that in the infant cereals from 0.05-0.49 mg/100 g. Amounts of non-α-tocopherols ranged from one to approximately three times the α-T content. In view of these low levels, the low α-T/PUFA ratios found and the importance of vitamin E in infant nutrition they concluded that infant formulas should be supplemented. Kanno et al. (37) found both α-T and γ-T in bovine milk fat. Mean values for α-T and γ-T over a 1-year period were 2.83 and 0.15 mg/100 g lipid, respectively. During the summer α-T and γ-T averages 3.38 and 0.18 mg/100 g lipid; in winter, 2.16 and 0.11. No other forms were found. Tsuga et al. (38) found the same forms in cow's milk and a similar change from winter to summer. α-T varied from 1.8 mg/100 g fat in February to 3.48 in July; in the same period, γ-T varied from 0.06 to 0.22.

Other Animal Products

Few other animal products have been examined for their tocopherol content, principally because the amounts are low and make little contribution to the diet. Ackman and Cormier (39) found small amounts of α-T in Atlantic cod, flounder, mackerel and lobster; this presumably enters the food chain by way of the algae and seaweed. The dark tissue of the winter cod had about five times as much as the white tissue (1.17 vs. 0.22 mg/100 g, fresh weight). Lobster tail and claw meat were the richest, with 1.67 mg α-T/100 g fresh weight. They also found that holding fish in the starving state decreased α-T, in some cases to zero.

Tsuga et al. (38) found α-T, γ-T and δ-T in white Leghorn eggs at 4.0, 2.1 and 0.1 mg/100 g lipid, respectively. Lard has been found to contain small variable amounts of tocopherol. Blattna et al. (17) analyzed two samples and found 7.0 mg α-T/100 g in one and only traces in the other. In our laboratory we have analyzed only one sample and found 1.2 mg α-T, 0.07 mg α-T-3 and 0.07 mg γ-T/100 g.

In summary, there has been a significant increase in recent years in our knowledge of the tocopherols in foods, particularly for the major sources such as seeds and vegetable oils. For some of these, consistent patterns are beginning to emerge. Refined and prepared foods are less predictable. These have tocopherol contents that depend on processing, treatment and formulation, and both identities and amounts vary greatly. The accumulation of a body of useful data will depend on the use of specific reproducible methods applied to adequately described samples. There is still much work to be done in this field.

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A Function for α-Tocopherol: Stabilization of the Microsomal Membrane From Radical Attack During TPNH-Dependent Oxidations[1]

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ABSTRACT

Events accompanying electron transport in the membrane fraction of liver and other tissues have led us to propose a specific function for α-tocopherol based on a sequence of biochemical changes we observed to occur in these membranes and on pertinent information from other laboratories. The activity of a membrane-bound enzyme system (TPNH oxidase) which involves transport of electrons from substrate to oxygen, has been shown to promote simultaneous formation of peroxide functions on the β position polyunsaturated fatty acids (PUFA) of phospholipids in the membrane. The phospholipid peroxides then undergo a chain cleavage reaction producing phospholipids containing a variety of carbonyl moieties in the β position. The process results in marked alteration of the membrane structure. During the overall reaction α-tocopherol present in the membrane is converted to a compound more polar than tocopheryl quinone and the conversion is dependent on the same enzymic factors promoting the phospholipid alterations. The membrane alteration process is enhanced in microsomes from animals fed diets containing relatively high levels of PUFA or diets low in α-tocopherol, and is diminished by low levels of dietary PUFA or relatively high levels of α-tocopherol. The experimental data indicate that enzymic electron transport associated with TPNH oxidation by the microsomal membrane involves free radical functions. The latter apparently can promote extensive peroxidative alterations of phospholipids that result in structural changes in the membrane unless adequate α-tocopherol is present in this organelle. This system appears to be part of the microsomal drug metabolizing system.

INTRODUCTION

It has been recognized for some time that diets containing significant quantities of polyunsaturated fatty acids (PUFA) but not containing an appropriate level of α-tocopherol can promote destructive processes in various animal tissues (1). Both the short and long term consequences of feeding such diets indicate that some metabolic system is significantly influenced by the relative amounts of polyunsaturated fat and α-tocopherol in the diet. Because of the properties of the substances involved, a number of investigators logically proposed that autoxidation of unsaturated fatty acids in tissues of animals deficient in tocopherol or other suitable antioxidant produce toxic substances and cause damage to lipoprotein structures which initiate the pathological changes seen (2-4). Our studies with in vitro systems indicate that peroxidation of lipid does indeed occur, however it appears not to be a random autocatalytic process, but one promoted by the highly localized production of free radicals formed as part of the mechanism of action of certain enzymes. When there is adequate tocopherol in the tissues to react with them, such radicals apparently produce very little alteration in the lipids of structures around the site of their origin. When the tocopherol level is inadequate, measurable damage may occur to cellular membranes as a result of ordinary enzymic activities. Such a localized mechanism for the production of cellular lesions also has the advantage of being able to account for the rather specific nature of the damage seen in tocopherol deficiency diseases, and, in addition, of providing an explanation for at least part of the metabolic turnover of α-tocopherol in normal animal tissues. The data to be presented will show that the activity of a

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TABLE I

Distribution of TPNH Dependent Phospholipid Oxidation Activity in Microsomes of Tissues of Various Species

Animal	Tissue	Specific activity[a]
Rat	Liver	64.2
	Muscle	15.1
	Brain	23.9
Human	Liver	28.6
	Muscle	9.3
	Brain	6.4
Rabbit	Liver	23.9
Chick	Liver	36.3
	Muscle	23.5
	Brain	6.4
	Kidney	17.1
Toad	Liver	35.4
	Muscle	38.1
Catfish	Liver	16.6
	Muscle	6.2

[a]Millimicromole of malondialdehyde detected by the thiobarbituric acid reaction per milligram microsomal protein.

constitutive enzyme system (TPNH oxidase) in animal tissues apparently catalyzes the production of radicals in sufficient concentration to cause a specific type of oxidative degradation of phospholipids in membranes of cells containing this enzyme system. The behavior of the TPNH oxidase system, present in the endoplasmic reticulum of liver and other tissues, will be shown to be sensitive to the levels of dietary PUFA and α-tocopherol which existed in vivo. The reaction rapidly metabolizes tocopherol to an unknown compound and causes extensive alteration of membrane structures in vitro. The latter process is accelerated in tissues from animals maintained under dietary conditions which are known to precipitate tocopherol deficiency disease. It would be an unusual coincidence, therefore, if the biochemical events to be described are not involved in the pathological changes seen in tissues of animals subjected to high PUFA-low tocopherol diets. These events represent the first example which demonstrates that the effects of dietary tocopherol and PUFA can be observed to converge upon the same enzymic system, and in which the enzyme system causes chemical alteration of these dietary components. We are proposing a function for α-tocopherol based on its behavior in the endoplasmic reticulum during the reaction described. This proposed function differs from the generally held antioxidant concept only in that it defines a specific metabolic system, capable of initiating lipid peroxidation, where tocopherol can exert its effect.

TABLE II

Comparison of TPNH Dependent Oxidative Chain Cleavage of 20:4ω6 and Associated Malondialdehyde Formation in Microsomes From Tocopherol Sufficient and Tocopherol Deficient Rats[a]

Dietary group	20:4ω6 Consumed, % of total	Malondialdehyde formed, mμmoles/mg/protein
Tocopherol sufficient	16	19
Tocopherol deficient	89	55

[a]Microsomes were isolated as described in the text from rats given the purified diet specified. The diet of supplemented rats contained 10 mg% α-tocopheryl acetate. The reaction systems were incubated for 45 min at 37 C.

MATERIALS AND METHODS

Chemicals

Materials for the purified diets were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, except for the stripped lard. The latter was purchased from Distillation Products Industries, Rochester, N.Y., as were also *d*-α-tocopherol and *d*-α-tocopherol-5-^{14}C-methyl. The following reagents and chemicals were obtained as indicated. TPNH and N-propyl gallate: Sigma Chemical Co., St. Louis, Mo.; diphenyl-*p*-phenylene diamine, aniline, diphenyl amine and 2-thiobarbituric acid: Eastman Organic Chemicals, Rochester, N.Y.; alumina: Bio-Rad Laboratories, Richmond, Calif.; N-methyl aniline hydrochloride: K and K Laboratories, Plainview, N.Y.; ADP: P-L Biochemicals, mIlwaukee, Wis.; Santoquin: Monsanto Chemical Co., St. Louis, Mo. All supplies related to gas liquid chromatographic analyses were obtained from Applied Science Laboratories, State College, Pa. Other chemicals used were of reagent grade quality.

Animals

Adult, male rats of the Sprague-Dawley strain were used in all experiments. The animals were fed a rat laboratory ration obtained from Rockland Laboratories, Monmouth, Ill., unless otherwise indicated.

Preparation of Microsomes

Microsomes were prepared from freshly isolated rat liver by homogenization in 0.15 M potassium phosphate buffer, pH 7.4 (1 g of liver to 5 ml of buffer) and centrifuging at 10,000 x *g* for 1 hr (4 C). The microsomal fraction was washed twice by resuspension in 0.15 M phosphate buffer and centrifuging at 100,000 x *g*. The final washed particles can be stored for several weeks as the sedimented pellet at -20 C. When ready for use, the pellet

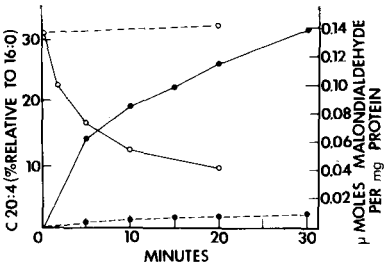

FIG. 1. Utilization of 20:4ω6 in microsomal phospholipid and formation of malondialdehyde during TPNH oxidation by microsomes. The composition of the reaction system and conditions of the incubation are given in the text. ○——○ 20:4ω6 content of microsomal phospholipids in experimental system; ○---○ 20:4ω6 content of microsomal phospholipids in control system; ●——● malondialdehyde formation in experimental system; ●---● malondialdehyde formation in control system.

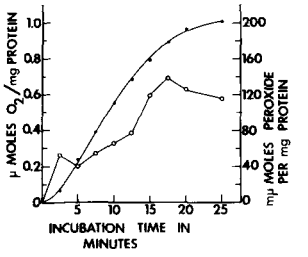

FIG. 2. Phospholipid peroxide formation and oxygen consumption during TPNH oxidation by liver microsomes. O_2 consumption was determined with 2 ml reaction systems while peroxide determinations were performed on 1 ml systems. The values used to plot the curves shown are those of the experimental system minus the control systems. In the control systems the values obtained for O_2 consumption were essentially negligible and the peroxide values did not change from their initial low value, indicating that incubation of microsomes per se does not contribute to the peroxide formation. See text for details. ●——● oxygen uptake; ○——○ peroxide formation.

was resuspended in 0.1 M tris-HCl buffer, pH 7.4, so that 1 ml of the suspension contained the microsomes from 1 g of liver. Microsomes from brain and kidney were prepared in the same way. Muscle microsomes were prepared by the method of Martonosi et al. (5) except that the particles were suspended in 0.1 M tris HCl buffer, pH 7.4, when resuspended. The protein content of enzyme preparations was assayed by the method of Lowry et al. (6).

Incubation System

The incubation systems had the following composition (except where indicated otherwise). Experimental: microsomes, 1 mg protein/ml of reaction system; tris-HCl buffer, 0.1 M (pH 7.4); ADP, 4 mM (Disodium salt; promotes the solution of $FeCl_3$ in aqueous system and is presumed to make the metal ion available to a binding site in a chelated form, Reference 15); $FeCl_3$, 0.012 mM; TPNH, 0.3 mM. Control: same as the experimental system except that no TPNH was added. Reaction volumes were from 1 to 10 ml. Incubations were carried out at 37 C in a shaking waterbath for 1 hr or as specified.

Assays for TPNH Dependent Oxidative Chain Cleavage of Phospholipid PUFA

Disappearance of Phospholipid Polyunsaturated Fatty Acids. Control and experimental reaction systems of 10 ml each were extracted at the end of a 1.0 hr incubation period by adding 15 vol of $CHCl_3$-CH_3OH (2:1) containing a known amount of an internal fatty acid methyl ester standard. After standing for 30 min, 1/5 vol of 0.9% NaCl was added and the two phases allowed to separate at 4 C. The chloroform layer was recovered and evaporated to dryness. The lipid residue was recovered. Total lipid phosphorus was determined (7) after decomposing a measured amount of the lipid in hot H_2SO_4. A sample of the lipid extract was taken for the preparation of methyl esters using BF_3-methanol. Gas liquid chromatography was performed at 185 C on a Perkin-Elmer model 881 equipped with a flame ionization detector and a 6 ft x ¼ in. O.D. column packed with ethylene glycol succinate, 12% on Chromosorb W, 60/80 mesh. Absolute quantities of each fatty acid present were calculated from the internal standard, and the extent of PUFA utilization determined by the difference in content between experimental and control.

Malondialdehyde Formation. This substance was determined after termination of the control and experimental reaction systems with 0.5 ml of 35% trichloroacetic acid per milliliter of incubation system. The procedure was the same as that of Ottolenghi (8).

Oxygen Consumption. For short term reaction, the oxygen uptake of 2 ml systems was determined over a period of 2-5 min, with an oxygen electrode. For longer incubation periods, a differential manometer was employed, using 5-10 ml reaction systems.

Peroxide Formation. The peroxide content of the reaction systems was determined by the procedure of Kokatnur et al. (9).

Turbidity Measurements. This assay was performed by observing the change in optical density of the reaction systems at 520 mμ (10).

Determination of Tocopherol Utilization During TPNH Dependent PUFA Utilization by Microsomes

Approximately 3 μc of *d*-α-tocopherol-5-^{14}C-methyl was dispersed in 0.05 ml of ethanol and

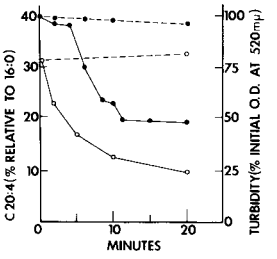

FIG. 3. Turbidity change in the microsomal suspension during TPNH oxidation accompanied by peroxidative chain cleavage of microsomal PUFA. ●——● turbidity of experimental system; ●—● turbidity of control system; ○——○ 20:4ω6 content of microsomal phospholipids in experimental system; ○—○ 20:4ω6 content of microsomal phospholipids of control system.

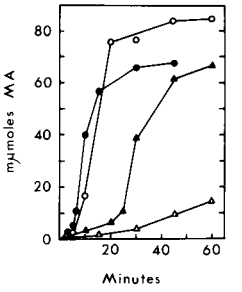

FIG. 4. Effect of increased dietary level of α-tocopheryl acetate on the formation of malondialdehyde (MA) by microsomal TPNH oxidase. See text for source of diet composition. Basal supplemented diet (E1) contained 10 mg of tocopheryl acetate per 100 g of diet. The number associated with each diet designation represents a multiple of the amount of tocopheryl acetate in the basal diet, e.g., E3 contained 30 mg/100 g diet and E5 contained 50 mg/100 g diet. Animals were on the designated diet 4 days. ● E1; ○ E3; ▲ E6; Δ E9.

0.5 ml of 16% Tween 80 in 0.9% NaCl and injected intraperitoneally. Three hours later the liver was removed from the rat and microsomes prepared as above. Control and experimental reaction systems were incubated for 45 min at 37 C and then the lipids were extracted with $CHCl_3$-CH_3OH (2:1) containing 30 mg each of α-tocopherol, α- tocopheryl quinone (11) and a mixture of dimer and trimer compounds (12), all of which serve as carriers. Purification of these three components was achieved by chromatography on alumina columns according to the procedure of Mellors and Barnes (13). The radioactivity of the recovered material was determined by liquid scintillation counting, corrected for quenching.

Standard Diets

To demonstrate the effect of dietary fat on the enzymic process, a purified diet used by Young and Dinning (14) was prepared in two ways: with the 3% lard and 3% cod liver oil as described, and without the lard and cod liver oil, but containing 1.3% linoleic acid instead, adjusting the carbohydrate content to compensate for the reduced fat content. All diets were fed ad lib. Addition of α-tocopheryl acetate was made as described in the Tables and Figures.

Hemolysis Assay for Radicals

Rat blood, freshly drawn by heart puncture, was centrifuged and the plasma and buffy coat removed. The erythrocytes were washed twice with approximately 2 vol of 0.9% NaCl. Packed red cells (0.1 ml/ml of the TPNH oxidase system) were used in the hemolysis assay. After incubation, the reaction systems were centrifuged to sediment ghosts and unhemolyzed cells and the intensity of the hemoglobin color was measured by determining the optical density of the supernate at 545 mμ.

RESULTS AND DISCUSSION

An interest in the biochemical function of α-tocopherol and two sets of observations by others led the authors to undertake an investigation of the relationship between α-tocopherol, membrane-bound oxidoreductase systems and the lipids which form part of the structure of those membranes. The first observations were those of Beloff-Chain et al. (16) who reported that the incubation of liver microsomes with TPNH, ADP and inorganic phosphate resulted in TPNH oxidation and an excessive amount of oxygen uptake, but no formation of ATP. The second set of observations were those of Hochstein and Ernster who determined that a substance with characteristics resembling malondialdehyde was produced during this reaction. They concluded that TPNH oxidation by liver microsomes was accompanied by lipid peroxidation (17) and subsequently determined that inorganic iron was involved in the process (18).

TPNH Dependent Disappearance of Microsomal PUFA

It was determined in our laboratory that the PUFA in the microsomal phospholipids were disappearing at a high rate in the reaction. The most abundant PUFA present (20:4ω6, comprising 15% to 20% of the microsomal fatty acids) rapidly fell to less than half of its original level (19). Figure 1 shows that within 20 min the 20:4ω6 level of the microsomes was reduced to one third of its initial value. Other PUFA present (20:5ω6 and 22:6ω3) showed similar decreases. During the reaction, the material reacting with thiobarbituric acid to form a pigment absorbing at 532 mμ accumu-

TABLE III

Disappearance of ^{14}C-labeled α-Tocopherol in Microsomes During the Oxidation of TPNH[a]

System	Specific activity of compounds isolated from microsomes, DPM/mg			Recovery of total counts in extracted lipid fraction, %
	Dimer-trimer	α-Tocopherol	α-Tocopheryl quinone	
Unincubated microsomes	57 ± 13	399 ± 18	66 ± 15	87 ± 2
Control	55 ± 11	388 ± 88	137 ± 74	81 ± 12
Experimental	80 ± 26	57 ± 24	231 ± 24	59 ± 10

[a]The control and experimental incubation systems were 10 ml in volume and were composed as described under Materials and Methods. Incubations were carried out in a shaking water bath at 37 C for 1 hr. At the end of that time the reaction system was extracted with $CHCl_3$-CH_3OH (2:1) containing known amounts of carrier α-tocopherol, α-tocopheryl quinone and a mixture of dimer and trimer compounds. The unincubated microsomes were extracted immediately after isolation from the liver. The total counts in the extracted lipids were determined and then the latter were fractionated on alumina as described in the text. The recovered fractions were analyzed by thin layer chromatography for purity and their specific activity determined. Values are averages of three experiments ± standard deviations.

lated. This product was shown to be malondialdehyde and is the only one of the many carbonyl products formed by the microsomal reaction which forms the pigment (20). It was also determined that malondialdehyde formation was always proportional to the total amount of PUFA utilized in the reaction. The results indicate that oxidative chain cleavage of PUFA was occurring. The reaction was shown to be enzymatic and, on reaching completion, consumed 1 mole of PUFA and 4 moles of O_2 for each mole of TPNH oxidized (21).

Phospholipid peroxides were shown to be intermediates in the reaction (Fig. 2). The peroxides accumulated during the early part of the reaction if the incubation medium is oxygenated (22) but form at lower levels when incubated in air. (Oxygen electrode experiments have shown the oxygen concentration drops to very low levels within minutes in systems incubated in air. Oxygen uptake by the reaction under these conditions is limited to the rate of diffusion of O_2 from ambient air into the incubation system) The level of peroxide accumulation is determined by the difference in the rate of formation and the rate at which the peroxides decompose to form malondialdehyde, phospholipid-bound aldehydes and other products (22). The structure of the microsomal membrane undergoes major physical change during the PUFA utilization which can be observed by measurement of the turbidity of the incubation system during the reaction (Fig. 3). The nature of this physical change is not known although electron photomicrographs indicated that the microsomal particles remained intact.

Distribution of the TPNH Dependent System in Tissues of Various Species

Although all of the studies in this report were done with rat liver microsomes, the phenomena described above are also present in other tissues in the rat and various other species. Table I lists the specific activity of the process in microsomes from several sources. In almost all cases the activity was highest in liver microsomes, and those from rat had the highest activity of any. There was also significant activity in skeletal muscle while whole brain had generally less activity.

Conversion of α-Tocopherol to a Polar Compound During the Reaction

The effect of dietary α-tocopherol on the activity of this enzymic system was determined. Microsomes from rats fed a diet containing 10 mg of α-tocopheryl acetate per 100 g of diet were observed to utilize 20:4ω6 and form malondialdehyde at a much slower rate than microsomes from rats on the same diet without tocopherol (Table II). The results suggested that α-tocopherol was interacting with the TPNH dependent PUFA peroxidizing system in some way that depressed the rate of the reaction. To test this possibility further, several groups of rats were placed on a diet which was supplemented with different amounts of α-tocopheryl acetate as described in Figure 4. Liver microsomes were prepared from animals in the various groups and assayed for the enzymic activity by determining the course of malondialdehyde formation. The results shown in Figure 4 clearly indicate that as the level of α-tocopheryl acetate in the diet increased,

TABLE IV

Conversion of ^{14}C-labeled α-Tocopherol to an Apparently Higher Polar Substance in Microsomes During TPNH Oxidation[a]

System	Specific activity of compound isolated from microsomes, DPM/mg				
	Dimer-trimer	α-Tocopherol	α-Tocopheryl quinone	Acidic methanol fraction	% Recovery of total counts in lipid fraction
Unincubated microsomes	22.7	203.1	56.0	62.8	88
Control	36.5	100.6	134.4	95.6	94
Experimental	17.6	11.5	142.4	491.8	69
Unincubated microsomes	35.9	260.1	112.5	112.6	85
Control	26.9	214.9	136.8	151.6	86
Experimental	34.1	37.4	106.5	420.1	64
Unincubated microsomes	40.4	330.4	167.0	262.3	91
Control	37.2	255.4	175.5	293.2	88
Experimental	46.8	26.5	148.0	914.7	73

[a]The composition of the reaction systems and conditions of incubation were as described in Table I. The extractions of the lipids and fractionation on alumina columns were as described in Table I and under Materials and Methods. The acidic methanol fraction was obtained by eluting the columns with 15% HCl in methanol after recovery of the other components was completed.

TPNH dependent cleavage of PUFA decreased but was not completely suppressed even by the highest dietary level tested (90 mg/100 g). The sigmoid shape of the progress curves suggested that tocopherol produced a lag period, the length of which was related to the level of supplementation of the vitamin and that the vitamin was preventing rapid oxidative chain cleavage of PUFA, at least initially, during TPNH oxidation.

Studies on metabolism of α-tocopherol by Wiss et al (23)and Krishnamurthy and Bieri (24) had suggested that this compound turned over rapidly in liver. In addition, Skinner and Alaupovic (25) reported that radioactive tocopherol given to rats appeared in liver microsomes as a metabolite which they designated as compound "O," and later suggested by Draper et al (26) to be a mixture of dimer and trimer. Since the enzymic activity we were investigating appears to be part of the drug metabolizing system (27), it seemed reasonable to consider the possibility that TPNH oxidation was promoting the production of oxygen radicals which could attack PUFA, but that the latter might be protected from such attach by α-tocopherol in an ordered membrane structure. In that case, α-tocopherol would be acting as a radical trapping agent and would undergo chemical alteration. Since such action was in agreement with the properties of the vitamin, we investigated the behavior of α-tocopherol in the microsome during TPNH oxidation.

Rats were injected intraperitoneally with ^{14}C-labeled α-tocopherol as described under Materials and Methods, and after allowing 3 hr for absorption, liver microsomes were prepared. These were incubated in control and experimental systems and then the lipids were extracted with $CHCl_3$-CH_3OH (2:1) containing known weights of α-tocopherol, α-tocopheryl quinone and a dimer-trimer mixture, all as carriers. In addition, unincubated microsomes were also extracted in the same way. After determining the amount of radioactivity in the extracts, the total lipids were fractionated on alumina columns and the three carrier substances were recovered. Recovery of these compounds was always higher than 95% and on thin layer chromatography (TLC) appeared as single spots. The specific activities of the compounds were determined. Table III shows that during TPNH oxidation in microsomes, about 85% of the radioactive microsomal tocopherol disappeared. The conditions of the incubation itself had little effect on microsomal tocopherol since the amount present in the control system was approximately the same as in unincubated microsomes; therefore, the loss of tocopherol in the experimental system microsomes was a consequence of TPNH oxidation. Although there appeared to be increased radioactivity in the quinone fraction, this was subsequently found not to be significant. Recovery of total counts in the lipid from the experimental system microsomes tended to be low, and since the recovery of the three carriers was essentially complete, it was assumed that some other radioactive substance or substances were still on the alumina columns.

Subsequent experiments proved that this

TABLE V

Inhibition of TPNH Oxidase-Catalyzed Hemolysis by Substances Known to React With Radicals[a]

Compound added to reaction system	Per cent inhibition of hemolysis
Diphenyl-*p*-phenylenediamine (8)[b]	93.4 ± 2.4
N-Propyl gallate (3)	92.2 ± 2.1
Diphenyl amine (7)	93.9 ± 0.8
Santoquin (6)	95.9 ± 1.2
Aniline (4)	88.3 ± 0.8
N-Methyl aniline (4)	94.2 ± 0.2

[a]The reaction systems were 1 ml in volume and their composition and conditions of incubation were as described in the text. One tenth milliliter of packed, washed erythrocytes were added to both control (without radical-trapping agent) and experimental (with agent) systems. Final concentration of inhibitors was 1 x 10^{-3} M.

[b]The number in parenthesis indicates the number of experiments done.

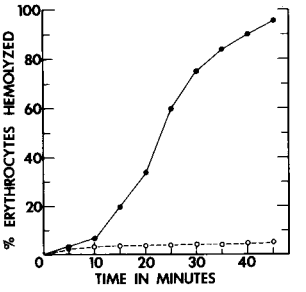

FIG. 5. Hemolysis of erythrocytes by a factor having properties of a free radical produced by the microsomal TPNH oxidase system. Control and experimental reaction systems of 1.0 ml volumes were used, each containing 0.1 ml of washed erythrocytes. The assay was performed as described in the text. ●——● experimental system; ○---○ control system.

was correct. Table IV shows results confirming the decrease in radioactive α-tocopherol in microsomes incubated with TPNH. In addition, after eluting the dimer-trimer group, α-tocopherol and α-tocopheryl quinone from the alumina, 15% HCl in methanol was passed through the column. Additional lipid was recovered by this procedure and its specific activity determined. The data in Table IV show that the material eluted from the column on which the experimental system lipid was fractionated contained considerably more radioactivity than the material from the columns on which the control system and unincubated microsome extracts were fractionated. These findings indicate that microsomal α-tocopherol is converted to a compound with chromatographic behavior of a polar lipid compound. The nature of the material eluted with acidic methanol is unknown, nor is it known if the radioactivity it contains is in a single compound since TLC shows the presence of several spots each of which contains some radioactivity. This, however, may have been the result of decomposition in the acidic methanol. Polar compounds eluted from alumina by acidic methanol were also observed by Chow et al (11) in the ether extract of acid-hydrolyzed rat urine after injection of ^{14}C-labeled α-tocopheryl quinone and ^{14}C-labeled α-tocopheryl hydroquinone. The compounds contained almost half of the total radioactivity in urine. These investigators concluded that the polar compounds they observed were degradation products produced by the acid hydrolysis of conjugated α-tocopheronic acid. In our studies, no acid hydrolysis of the microsomal lipid was performed before column chromatography. As suggested above, it is possible that elution with methanolic HCl might cause degradation of a conjugate on the column if such a compound were extracted from the microsomes by the procedure used. Our recent experiments have shown that most of the α-tocopherol in the microsomal system was metabolized during the first 2-3 min of the TPNH oxidase reaction, during which time only a small amount of the microsomal PUFA has been consumed.

The results of our studies with the behavior of ^{14}C-labeled α-tocopherol in microsomes during TPNH oxidation suggested that free radicals produced by the enzyme system may attack α-tocopherol in the microsomal membrane under certain conditions, promoting the formation of the polar product, and, as a result, the tocopherol content of the membrane rapidly falls. When that occurs, the attack on PUFA must then be greatly enhanced. This concept would adequately explain the lag periods observed in microsomes from animals supplemented with higher levels of α-tocopherol (Fig. 4).

Erythrocytes as Indicators of Radical Formation

Our attempts to demonstrate by electron spin resonance studies the formation of radicals during the reaction were negative. This may have been due to very short half lives of the radicals involved or for reasons we have explained in more detail in a recent report (20). However, the fact that peroxidative cleavage of PUFA is prevented by a wide variety of structurally unrelated radical trapping agents, and that it promoted rapid sulfite oxidation (21), indicated radical involvement. We recently determined that washed erythrocytes can be used to detect the presence of a factor produced by this enzymic reaction which

TABLE VI

Effect of Dietary Lipid on the TPNH Oxidase-Catalyzed Chain Cleavage of Phospholipid Polyunsaturated Fatty Acids in Liver Microsomes[a]

Dietary group	Specific activity[b]	
	3% CLO-3% lard[c]	1.5% Linoleic acid[d]
Tocopherol deficient	56.7 ± 19.0	11.5 ± 2.7
Tocopherol sufficient	5.1 ± 3.5	5.1 ± 0.9

[a]The diets were identical other than for the difference in the amount and type of fat contained (see text). The carbohydrate content was adjusted as required.
[b]mμmoles of malondialdehyde formed at 532 mμ/mg protein.
[c]Values are averages of nine experiments.
[d]Values are averages of 16 experiments.

attacks the erythrocyte membrane, causing rapid hemolysis, but is prevented from doing so by a number of radical trapping agents. The factor also exists only when the enzyme system is functioning, disappearing immediately when the enzyme is inactivated, indicating a very short half life. The factor is, therefore, if not in fact a radical, a highly reactive chemical species with properties of a free radical. Figure 5 shows the result of incubating rat erythrocytes with the TPNH oxidase system. Complete hemolysis of 0.1 ml of packed red cells was promoted by a 1.0 ml reaction system in less than 1 hr. Essentially no hemolysis occurred in the control system.

Table V gives data which support the assumption that the factor generated by the TPNH oxidase system is a radical. Six different substances known to be radical trapping agents or substances capable of reacting with radicals were tested for their ability to prevent hemolysis during TPNH oxidase activity. All were approximately 90% effective at 1 x 10^{-3} M final concentration in protecting erythrocytes from hemolysis, presumably by their own reaction with the radicals. In addition, treating the rats which donated erythrocytes with an antioxidant yielded red cells which did not hemolyze in the reaction. Similarly, treating rats from which microsomes were prepared gave a microsome preparation which did not hemolyze normal red cells during TPNH oxidation.

Effect of Dietary Lipid on the TPNH Dependent Chain Cleavage of PUFA

An additional dietary factor other than the level of α-tocopherol was also found to affect the reaction promoting cleavage of microsomal PUFA. Rats fed a diet containing significant amounts of fat, especially polyunsaturated fatty acids yielded liver microsomes in which the reaction is very active. Table VI shows how changing the dietary fat content and the level of tocopherol supplementation affect the system. The most active microsomes in this study were those from tocopherol deficient animals whose diet contained 3% cod liver oil and 3% lard. Animals fed the same tocopherol deficient diet but in which the 6% fat mixture of cod liver oil and lard was substituted by 1.5% linoleic acid yielded microsomes only about one fifth as active even though liver tissue from both sets of animals contained essentially the same amount of phospholipid bound arachidonic acid, which is the chief substance of the reaction. Supplementation of both diets with α-tocopherol resulted in low activity in both groups. There are several possible explanations for this effect: diets which contain higher levels of fats, especially PUFA, may facilitate the incorporation of the PUFA into sites in the microsomal membrane where they are susceptible to radical attack while diets promoting in vivo synthesis of PUFA may result in a different distribution; the TPNH oxidase system which promotes radical formation may be induced by diets with higher levels of fats; radical production by the TPNH oxidase system is promoted only by certain types of dietary fatty acids. All of these possibilities can be tested.

The significance of these studies lies in their support of the concept that α-tocopherol may exert its major biological effect by acting as a trapping agent for radicals produced by the normal activity of certain membrane-bound enzymes. Such radicals may be essential to the mechanism of the reaction and yet have the potential for initiating peroxidation of polyunsaturated fatty acids in phospholipids adjacent to the enzyme in the membrane unless α-tocopherol is present to inhibit the process. One would expect tocopherol to be consumed during such activity and the data contained in this report shows that it is. An end product of α-tocopherol metabolism in animals has been shown to be α-tocopheronolactone (28) but the sequence of events which produces this substance is not known. Because of this and the

fact that the identity of the tocopherol metabolite observed in our studies is unknown, any relationship between the two processes cannot be assessed.

According to Krishnamurthy and Bieri (24) the total amount of radioactivity excreted in the urine of the rat over a 21-day period after injecting ^{14}C-labeled α-tocopherol was only about 1% of the injected dose, while Simon et al. (28) observed 30% excretion of the injected radioactivity in urine over a similar period of time in the rabbit. Bieri believes that the route by which labeled tocopherol is administered is important in the distribution of the vitamin and, therefore, to the rate of formation of radioactive metabolic products. This certainly seems likely. Our own studies with ^{14}C-labeled α-tocopherol were done by intraperitoneal injection. In our studies the effects of α-tocopherol on the enzymic system which we have described are the same whether tocopherol is given in the diet or by injection. The route of injection may determine the proportional distribution of the vitamin among the organs, but it probably does not influence the site within those organs where the tocopherol molecules will be situated except perhaps in the case of unusually large doses.

The nature of the radicals which appear to be produced by the enzyme is unknown, although we believe we have eliminated the superoxide anion [$\cdot O_2^-$] (20). Since the system involved is associated with the TPNH dependent drug metabolizing system, it is possible that the radicals being produced are those functioning in hydroxylation reactions. It is interesting that Lin and Chen have recently shown that in some hydroxylation reactions the peroxide of the substance forms first, and then this peroxide intermediate is reduced to the hydroxylated compound (29). It appears possible that our observations on TPNH dependent PUFA degradation are manifestations of the drug metabolizing system functioning without a suitable substrate since Orrenius et al. (27) and Slater (30) have shown that drugs which are hydroxylated by the TPNH dependent microsomal system decrease lipid peroxidation when added to the reaction. Gram and Fouts have shown that lipid peroxidation associated with the drug metabolizing system could be controlled by adding α-tocopherol to the reaction (31). These findings together with the results presented in this report suggest that a role for tocopherol in the liver microsome is to prevent excessive damage to the phospholipids in endoplasmic reticulum when the TPNH oxidase system is generating strongly oxidizing radicals for reactions such as sterol hydroxylations, desaturation of fatty acids, squalene hydroxylation and the detoxification of drugs and other organic substances such as carbon tetrachloride. The metabolism of carbon tetrachloride by liver microsomes has been shown to promote lipid peroxidation in these particles (32) and results in hepatic injury in the intact animal characterized by a loss of phospholipid PUFA (33). Further evidence that tocopherol is required to maintain the structure of the endoplasmic reticulum was given by Molenaar, Hommes, Braams and Polman, who demonstrated that in human beings suffering from abetalipoproteinemia, tocopherol deficiency develops and the endoplasmic reticulum disappears from the cells of the intestinal mucosa. Treatment of these patients with α-tocopherol resulted in the restoration of the normal endoplasmic reticulum structure (34).

It appears possible that erythrocytes may be a useful tool in searching for enzymes whose mechanisms of action produce potentially damaging factors (such as free radicals). Pertinent to this view is the fact that Goldstein and Balchum (35) have shown that an activated form of oxygen (ozone) is capable of producing fragility changes in erythrocytes. However, the factor produced by the TPNH oxidase reaction causes complete hemolysis of erythrocytes within minutes. Neither the individual reactants nor the products of this reaction cause this hemolysis. The hemolysis occurs only when the enzyme is actively functioning. The hemolyzing agent, therefore, must be a component which reacts readily with membrane lipids and has a very short half life. These findings, together with the protecting effect of free radical trapping agents and the fact that the enzyme is part of the mixed function oxidase complex, is strongly suggestive of an enzyme-catalyzed production of radicals.

The results of the studies presented can explain why structurally unrelated antioxidants may be substituted for tocopherol and why some are more effective than others, i.e., those which can be transported in sufficient quantities to the proper metabolic sites in membranes. None, in the long run, seem to have the effectiveness of tocopherol and this is probably a reflection of a molecular structure well suited for the purposes required.

If damage to cellular structures in tocopherol deficiency disease is due to attacks on structural lipids caused by radicals produced normally by the activity of certain enzymes, then the various animal species must have quite different amounts of such radical producing enzymes in various tissues or of ability to

transport dietary tocopherol to those sites of activity, or both, in order to account for the differing degrees of intensity and specificity of the lesions produced.

We have observed that the lipid products of the radical attack on microsomal phospholipids can polymerize which suggests that they may be related to the formation of ceroid pigments seen in tocopherol deficient animals and in aging humans, especially since the altered lipids exhibit an excitation-emission spectrum similar to that of the pigments. Chio and Tappel have shown that malondialdehyde and various amino acids form products with properties resembling those of age pigments (36).

The fact that membranous subcellular particles are susceptible to lipid peroxidation when exposed to a variety of substances and physical conditions has been well established. These include hemoproteins (37), carbon tetrachloride (32), ascorbic acid (8), chaotropic agents (38), light (39) and x-rays (40). It is, therefore, not surprising that microsomal membranes will undergo significant alteration if they contain enzymes whose activity initiates peroxidation of constitutive phospholipids unless adequate amounts of an antioxidant are present.

If this kind of interaction between certain enzymes and phospholipids in tocopherol deficient tissues represents the primary lesion of this nutritional disorder, it remains to be determined how such membrane changes can result in the severe cellular damage observed in rabbit muscle, chick cerebella, rat testes, etc. In this regard, it is interesting to note that malondialdehyde has been shown to inhibit DNA replication, protein synthesis and cell division in fibroblast cultures (41) and both malondialdehyde and peroxidizing linolenate cause inactivation of ribonuclease A and other enzymes, especially those with essential sulfhydryl groups (42).

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Studies of Differential Turnover of Palmitoyl and Stearoyl Species of Glycerophosphatides Using Labeled Unsaturated Acids[1]

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ABSTRACT

Normal and bile fistula rats were injected with 1-^{14}C-linoleate and arachidonate as albumin complex and the glycerides and glycerophosphatides of the liver and bile were isolated at various time intervals. The distribution of radioactivity among the individual molecular species was determined by thin layer and radio gas chromatography and specific enzymic hydrolyses. At 30 min after administration of linoleate 30% of the radioactivity in liver was in lecithins and 8% in cephalins, while at 120 min 48% was in lecithins and 16% in cephalins. After arachidonate, 58% and 64% of the counts were in lecithins and 12% to 13% in the cephalins at the above periods of sampling. The specific activity of the palmitoyl linoleoyl lecithins and cephalins was two to three times higher than that of the corresponding stearoyl linoleoyl species, which was of the same order but much lower magnitude than found previously for lecithins using labeled phosphate and choline. The palmitoyl and stearoyl species of arachidonoyl lecithins possessed equal specific activities, in sharp contrast to previous findings with radioactive phosphate, which showed a 12 times higher specific activity for the palmitoyl arachidonate. The palmitoyl arachidonoyl cephalins had two to three times greater specific activity than the corresponding stearoyl species in agreement with previous work using labeled phosphate. The distribution of radioactivity suggests that the arachidonate was incorporated into the lecithins largely via acyl transfer, while the linoleate contributed to both acyl transfer and de novo synthesis. Interpretation of the mechanism of uptake of these acids into the cephalins awaits further studies.

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INTRODUCTION

Although it has been well established (1-3) that there exist marked differences in the metabolic activity between 1-palmitoyl 2-linoleoyl and 1-stearoyl 2-arachidonoyl lecithins of rat liver, it has not been possible to decide unequivocally whether these discrepancies are due to the saturated or the unsaturated acids in these molecules (1-5). A similar uncertainty exists regarding the metabolic activity of other molecular species of lecithins and related phosphatides, such as the phosphatidyl ethanolamines. This has been due to the inability to effectively resolve these phospholipids and to trace their origin to the responsible metabolic pathway.

Since the dienoic and polyenoic fatty acids in the glycerophosphatides occur mainly in the 2 position and are paired with the saturated acids in the 1 position, an analytical scheme was envisaged which could assess the metabolic activity of any of the homologous pairs rapidly and accurately. It involved the introduction of the appropriate ^{14}C-labeled unsaturated fatty acid into the test system and a combined thin layer and radio gas chromatographic analysis of the labeled diglyceride moieties of the glycerophosphatides. From the measurements of the mass and radioactivity specific activities could be calculated and the relative turnover rates of the species assessed.

This report demonstrates the practical feasibility of the analytical system and suggests that the metabolic activity of a glycerophosphatide species is related to both component acids and is determined by the mechanism of biosynthesis of the species.

MATERIALS AND METHODS

Labeled Acids

1-^{14}C-linoleic acid (8-12 μc/μM) was purchased from New England Nuclear Corp., Boston, Mass. According to the distributors it had a radiochemical purity of greater than 98%. 1-^{14}C-arachidonic acid (53 μc/μM) was obtained from Applied Science Laboratories, Inc., State College, Pa. It contained a minor propor-

tion of unspecified *trans*-isomers. Argentation thin layer chromatography (TLC) of the methyl esters showed that over 99% of the counts resided in the dienoic and tetraenoic subfractions, respectively. Furthermore, radio gas chromatography of the methyl esters of these fatty acids revealed the activity in single peaks corresponding to linoleic and arachidonic acids.

Injection Solutions and Animals

An aliquot of the undiluted labeled acid (10 μC) was treated with a slight excess of dilute sodium hydroxide (0.13 ml) and the salt was complexed with 0.35 ml of bovine serum albumin in physiological saline to give a solution of 5 moles of acid per mole of albumin. Bovine serum albumin was purchased from Sigma Chemicals Co., St. Louis, Mo., and Pentex Biochemicals, Kanakee, Ill. The solution (0.5 ml) was injected via the jugular vein into male rats under light ether anesthesia. After injection the animals were allowed to recover. The rats (325-350 g) were of the Wistar strain and had been maintained on Purina Chow for seven days prior to the experiment. In a few instances bile was collected for 120 min by means of bile fistulae (6) introduced just before injection. Two rats were used for each time period in the linoleate experiments and one rat for each time period in the arachidonate experiments.

Isolation of Phospholipids

At suitable time intervals, the animals were bled from the abdominal aorta. The livers were rapidly excised and rinsed with cold saline. The liver and bile lipids were extracted according to the method of Folch et al. (7). The glycerophosphatides were isolated by TLC on silica gel G (Merck and Co.) using preparative plates (20 x 20 cm, 0.5 mm thick layers). The phosphatidyl ethanolamines were obtained with chloroform-methanol-water (65:25:4 v/v) and the phosphatidyl cholines with chloroform-methanol-ammonia (65:35:5 v/v) as the developing solvents (8). The purity and identity of the phospholipids so obtained was confirmed by TLC with chloroform-methanol-acetic acid-water (25:15:4:2 v/v) and by specific staining (9).

Resolution of Molecular Species of Glycerophosphatides

The molecular species of the phosphatides were resolved in the form of their diglyceride acetates following digestion with phospholipase C alone (lecithins) or with phospholipase C plus sphingomyelin (cephalins) and acetylation, as previously described (10,11). Diglyceride acetates of uniform degree of unsaturation were isolated by silver ion TLC, and the diglyceride classes were resolved on the basis of molecular weight by gas chromatography (10).

Determination of Specific Radioactivity

For this purpose the diglyceride acetates of uniform degree of unsaturation were examined further in a radio gas chromatograph equipped with a device for a continuous monitoring of radioactivity in the effluent stream of the column. The gas chromatograph was a modified Barber-Colman 5000 Series instrument. The working conditions were as described for studies on intact ^{14}C-labeled triglycerides (12). Both mass and radioactivity were measured quantitatively in relation to radioactive trioctanoin used as internal standard. The specific activities (dpm/μM) of the individual molecular species were calculated from the proportions of the mass and radioactivities as determined in the radio gas chromatograph after correction for the split ratio between the hydrogen flame and the radioactivity detectors. Alternatively, the specific activity of known weights of purified diglyceride acetates was obtained by counting in the scintillation spectrometer (Nuclear Chicago Corporation, 720 Series). The values obtained in this manner were in good agreement (± 10%) with those obtained using radioactive trioctanoin and radio gas chromatography as outlined previously (12). The counts were corrected to 100% efficiency by the channel ratio method. The weights of the purified diglycerides were obtained by gas chromatography in the presence of internal standard (10).

RESULTS

It was found necessary to inject intravenously about 10 μc of each fatty acid to obtain sufficient radioactivity in the liver lipids for a separate radio gas chromatographic assessment of the palmitoyl and stearoyl species of the glycerophosphatides. Judging from the recovery of counts after injection of equal doses of labeled material, the arachidonic acid was taken up by the liver about two times as readily as the linoleic acid. Furthermore, the bulk of the arachidonate appeared in the glycerophosphatides at all times of sampling, while the linoleate was incorporated initially to a greater extent in the triglycerides. The differences in the total uptake and distribution of these unsaturated acids in the rat liver lipids are shown in Table I. At 30 min approximately 60% of the counts from linoleate were localized in the neutral lipid fraction and 40% in the phospholipid fraction. By 120 min, the bulk of the activity (70%) was found in the phospho-

TABLE I

Incorporation of 1-^{14}C-Linoleic and 1-^{14}C-Arachidonic Acids Into Lipid Classes of Rat Liver[a]

Lipid fraction	1-^{14}C-Linoleate				1-^{14}C-Arachidonate			
	30 Min		120 Min					
	Expt. 1	Expt. 2	Expt. 1	Expt. 2	5 Min	15 Min	30 Min	120 Min
Neutral lipid	60.2	55.6	27.7	31.9	35.6	34.5	23.9	16.1
Phospholipid	39.8	44.4	72.3	68.1	64.4	65.5	76.1	83.9
Cardiolipin	0.5	0.5	1.4	1.0	---	---	---	---
PE[b]	8.1	8.5	18.7	14.0	10.4	11.0	12.6	12.6
PC	29.3	33.1	48.4	49.1	49.0	49.3	57.6	64.0
PS, PI, Sph, LPC	1.9	2.3	3.8	4.0	5.0	5.2	5.9	7.3

[a]Values given as per cent of total counts incorporated. Total disintegrations per minute incorporated per liver at the various times ranged from 2.07-3.21 x 10^6 (linoleate) and 3.76-5.99 x 10^6 (arachidonate), which represented 9.3-14.5% and 16.9-27.0% of the administered radioactivity, respectively. The mass proportions of various lipid classes were of the order reported previously (9,14).

[b]Abbreviations: PE, phosphatidyl ethanolamine; PC, phosphatidyl choline; PS, phosphatidyl serine; PI phosphatidyl inositol; Sph, sphingomyelin; LPC, lyso phosphatidyl choline.

lipids with the remaining 30% residing in the neutral lipids which is in general agreement with previous work (13). At all times, the phosphatidyl cholines contained about three fourths and the phosphatidyl ethanolamines nearly one fourth of the counts in the total phospholipid fraction. Phosphatidyl serine, phosphatidyl inositol and sphingomyelin together with lysophosphatidyl choline contained only minor amounts of the radioactive linoleate again in accordance with earlier work (14). The rapid and preferential incorporation of arachidonic acid into the phospholipids was apparent from the earliest samplings (5 min), and was in agreement with previous work with ^{14}C-labeled arachidonate (15). At no time did the distribution of radioactivity from either acid approximate the mass proportions of the different phospholipid classes in the rat liver.

The mass proportions of the fatty acids of the lecithins and cephalins agreed closely with those reported in the literature (2-4) and remained essentially unchanged during the experimental period. Table II gives the molecular weight distribution of the diglyceride acetates derived from the purified liver phospholipids obtained from a series of animals. Only minor variations are seen in the proportions of the various molecular weight classes in each glycerophosphatide when the animals are maintained on the same diet. However, the liver lecithins differ greatly from the cephalins in the ranges of the molecular weights represented. In comparison to the lecithins, the cephalins contain about one half the species with carbon number 36, two thirds those with 38, three halves those with 40 and double the amount of species with carbon number 42, as also noted by Wood and Harlow (16). Unlike liver, the biliary lecithins contained mainly carbon numbers 36 and 38, which was anticipated from the fatty acid analyses of Balint et al. (17).

For analysis of the specific activities of individual molecular species, the dienoic and tetraenoic diglyceride moieties of the glycerophosphatides were isolated by silver ion TLC. These species represented over 70% and 90% of the radioactivity of labeled linoleate and arachidonate, respectively. The dienes represented 28% and 70% of the mass of liver and bile lecithins. There were only small amounts of tetraenoic lecithins in bile. The mass of tetraenes represented 41% of the lecithins and 51% of cephalins, which was in agreement with

TABLE II

Carbon Number Distribution of Rat Liver Phospholipids[a]

Carbon number[b]	Phospholipids, mole %		
	Liver PC[c]	Liver PE	Bile PC
34	0.7 ± 0.1	---	1.2
36	17.9 ± 1.6	8.7 ± 1.1	62.6
38	34.7 ± 1.3	22.5 ± 0.8	29.6
40	41.6 ± 2.6	59.6 ± 2.2	6.6
42	5.0 ± 0.4	9.2 ± 0.8	---

[a]The values for liver PC are means ± standard deviation for seven rats, those for liver PE are means ± standard deviation for nine rats. Values for bile PC are averages of two rats.

[b]Refers to diglyceride acetates used for gas chromatography and includes the number of acyl carbon atoms in the molecule plus 2 (acetate).

[c]Abbreviations: PC, phosphatidyl choline; PE, phosphatidyl ethanolamine.

TABLE III

Specific Activity of Dienoic Glycerophosphatides of Rat Liver After Injection of 1-^{14}C-Linoleic Acid[a]

	PC, dpm/μmole			PE, dpm/μmole		
Minutes	34[b]	36[b]	Total	34	36	Total
Liver						
30	10,170	5,350	7,410	10,110	4,040	7,220
30	7,870	3,450	5,510	9,890	3,910	7,050
120	17,210	6,940	11,860	22,700	9,540	16,400
120	16,820	7,410	11,790	21,700	7,430	14,980
Bile						
120	20,390	15,810	19,970			

[a]Two rats were used for each time interval. Each pair of glycerophosphatides was obtained from the same liver.

[b]Carbon No. 34 and 36 designate the number of acyl carbons in the phosphatide molecle and are essentially palmitoyl-linoleoyl and stearoyl-linoleoyl, respectively, containing trace amounts of other species (16:0, 20:2; 14:0, 22:2; etc.). The dienoic fractions isolated at 30 and 120 min contained an average of 2.2% and 4.7% (PC) and 0.5% and 1.5% (PE), respectively, of the total administered radioactivity.

earlier work (4,18). Complete accounts of the molecular species of rat liver lecithins (19) and cephalins (B.J. Holub and A. Kuksis, in preparation) have been presented elsewhere. Table III gives the specific activities of the isolated dienoic species derived from the liver glycerophosphatides 30 and 120 min after injection of 1-^{14}C-linoleate. At 30 min, the dienoic lecithins and cephalins had approximately equal specific activities whereas at 120 min the specific activity of the cephalins exceeded that of the lecithins in confirmation of the findings of Balint et al. (5). At 120 min, the specific activity of the dienoic lecithins of bile was nearly twice that of the liver lecithins. If allowance is made for the difference in the total radioactivities administered, the specific activities for the bile lecithins are seen to be in the range reported by Balint et al. (5).

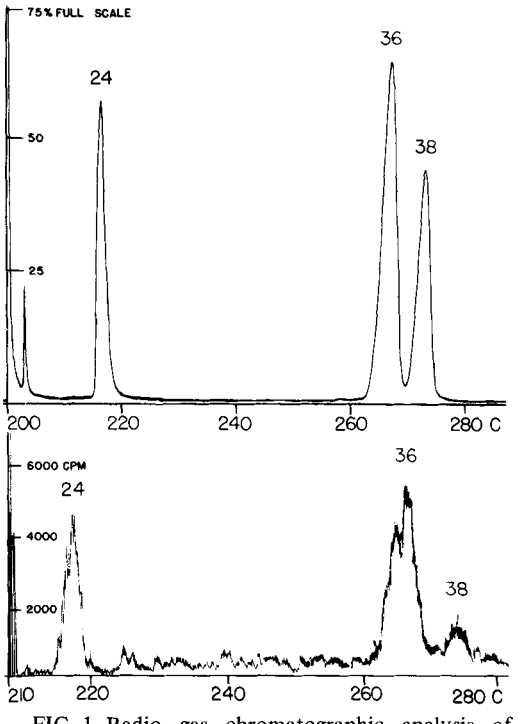

FIG. 1. Radio gas chromatographic analysis of dienoic palmitoyl and stearoyl lecithins. Upper print: Mass analysis. Lower print: Radioactivity analysis. Peak 24 represents glycerol 1-^{14}C-trioctanoate, internal standard. Other peaks as explained in Table II.

The relative specific activity of the palmitoyl-linoleoyl and stearoyl-linoleoyl species was ascertained by means of radio gas chromatography of the isolated dienoic diglyceride acetates. Figure 1 shows the mass (upper tracing) and radioactivity (lower tracing) obtained from such analyses on a sample of liver lecithin labeled with linoleate. It can be seen that much less activity was found in the stearoyl-linoleoyl pair than would have been expected from the relative masses of the palmitoyl and stearoyl species. This indicated that radioactive linoleate was incorporated preferentially into the palmitoyl lecithins. Table III shows that in a series of experiments at 30 and 120 min, the palmitoyl-linoleoyl species of the liver lecithins and cephalins consistently showed a specific activity two to three times that of the corresponding stearoyl-linoleoyl species. The palmitoyl linoleoyl lecithins of the bile showed a specific activity 1.3 times that of the stearoyl homologues, although these dienes were comprised largely of the palmitoyl linoleoyl pair.

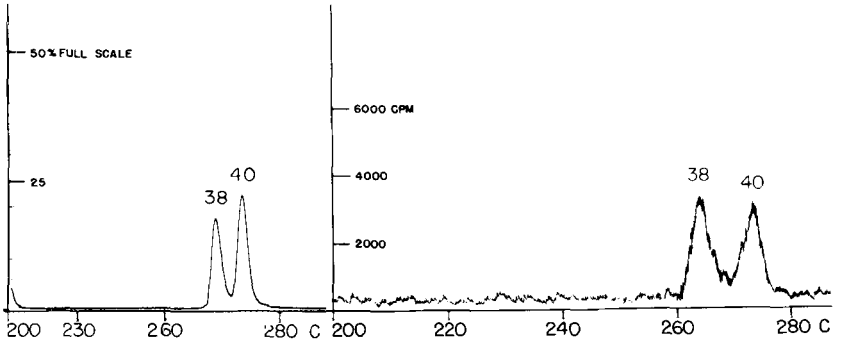

FIG. 2. Radio gas chromatographic analysis of tetraenoic palmitoyl and stearoyl lecithins. Left print: Mass analysis. Right print: Radioactivity analysis. Peaks as explained in Table II.

The purified dienoic and tetraenoic diglyceride acetates were also subjected to transmethylation and the derived fatty acid methyl esters examined by radio gas chromatography. At 120 min, 10-12% of the radioactivity recovered from 1-^{14}C-linoleate in the isolated dienes was found in the palmitic, and about 1% in stearic, with the remainder in linoleic acid. The data in Table III have been corrected for this by subtraction. Unlike linoleate, insignificant amounts of 1-^{14}C-arachidonate were utilized for fatty acid synthesis at these times, and no corrections were necessary.

Table IV shows that at all times studied, the specific activity of the total arachidonoyl lecithins was approximately twice that of the corresponding cephalins in agreement with the in vitro studies of Possmayer et al. (20), who found tritiated arachidonic acid to be preferentially incorporated into lecithin. Figure 2 shows the mass (left tracing) and radioactivity (right tracing) of a tetraenoic lecithin fraction labeled with arachidonate. Contrary to the observations with linoleate, significant activity was found also in the stearoyl species, and the relative specific activities of the palmitoyl and stearoyl arachidonates were approximately equal. From Table IV it can be seen that the radioactivities of the tetraenoic palmitoyl and stearoyl lecithins approximated their mass distributions. Of the tetraenoic cephalins, the palmitoyl species consistently showed a specific acitivity some two to three times that of the corresponding stearoyl species.

DISCUSSION

At least three different pathways are thought to be of importance for the incorporation of long chain fatty acids into liver phospholipids. One of these involves de novo synthesis via phosphatidic acid (21), another a stepwise methylation of phosphatidyl ethanolamine to phosphatidyl choline (22). A third pathway depends on the acylation of lysophosphoglycerides (23). Recent studies in vivo (3-5) have

TABLE IV

Specific Activity of Tetraenoic Glycerophosphatides of Rat Liver After Injection of 1-^{14}C-Arachidonic Acid[a]

	PC, dpm/μmole			PE, dpm/μmole		
Minutes	36[b]	38[b]	Total	36	38	Total
5	9,150	10,100	9,730	9,230	2,700	4,260
15	9,000	10,950	10,200	9,880	4,200	5,750
30	16,050	15,020	15,410	16,780	5,450	8,150
120	24,340	19,120	21,470	16,980	9,070	11,340

[a]Each pair of glycerophosphatides was obtained from the same liver. The ratio of the specific activities of total PC/PE at 5, 15, 30 and 120 min were 2.28, 1.77, 1.89 and 1.89, respectively.

[b]Carbon numbers designated as in footnote to Table III. Carbon No. 36 and 38 are essentially palmitoyl-arachidonoyl and stearoyl-arachidonoyl, respectively, containing trace amounts of other species. The tetraenoic fractions isolated at 5, 15, 30, and 120 minutes contained 8.3%, 10.1%, 14.6% and 17.3% (PC) and 1.8%, 2.3%, 3.2% and 3.4% (PE), respectively, of the total administered radioactivity.

suggested that the metabolic processes involved in the biosynthesis of the individual molecular species of the glycerophosphatides are determined both by their degree of unsaturation and also by the chain length of the saturated acid which they contain. On the basis of in vitro studies (18,24) it has been claimed that the phosphatidic acid pathway is highly operative in the formation of the monoenoic and dienoic species of phosphatidyl choline and phosphatidyl ethanolamine, whereas the more polyunsaturated molecules arise mainly via acylation of endogenous lysophosphatides. The quantitative importance of these pathways in vivo, however, has not been appraised.

Previous observations on the labeling of lecithins using 1,2-^{14}C-choline (2) and P^{32} (3) showed the palmitoyl linoleoyl species to have an average specific activity ten times that of the stearoyl linoleoyl species. Since a ratio of specific activities of 2.5 and not 10 was noted in this study, it would appear that the two species were not formed exclusively via de novo synthesis. If both species were formed via acyl transfer, a ratio of 0.6 would have been expected for their specific activities. Such a prediction can be made since the 1 position of our lecithin preparation contained equimolar concentrations of palmitate and stearate as did the endogenous lysolecithins of rat liver (25). From the nearly equal acyltransferase activities given by Brandt and Lands (26) for the 1-palmitoyl and 1-stearoyl lysolecithins and the knowledge of the relative concentration of the two linoleoyl species in liver lecithin, we would expect the palmitoyl linoleoyl species to have a specific activity 0.6 times that of the stearoyl homologue. The experimental value of 2.5 suggests that the two linoleoyl species are also not being formed equally by acyl transfer. Other investigators (27) have suggested that rat liver lecithins containing palmitic and linoleic acids are formed mainly via CDP-diglyceride. This is in apparent agreement with the work of Trewhella and Collins (3) who showed the palmitoyl linoleoyl lecithin to have the highest specific activity of all liver lecithins after administration of P^{32}. Furthermore, the studies of Akesson (28) showed the dienoic diglycerides of rat liver to be essentially of the palmitoyl linoleoyl type. These results suggest that the palmitoyl linoleoyl lecithin was derived via de novo synthesis. If the stearoyl linoleoyl homologue in our study were also being made exclusively de novo, it should have reached a specific activity only 10% that of the palmitoyl species. The fact that its specific activity was 40% that of the palmitoyl species suggests that some 75% (40-10/40 x 100) of the stearoyl homologue could have been formed via acyl transfer. Although methylation of phosphatidyl ethanolamine is of significance in the liver (22) it appears to be more active for the hexaenoic species (4,29).

Using P^{32} in vivo (3,4) and ^{14}C-glycerol in vitro (18) and in vivo (30) several workers have found a relatively low incorporation of the label into the arachidonoyl lecithins, which suggests that the phosphatidic acid pathway makes only a minor contribution to the synthesis of these species. Using P^{32} (3) it has been found that the palmitoyl arachidonoyl lecithins show a specific activity some 12 times greater than the corresponding stearoyl species. The latter findings are in contrast with our work with 1-^{14}C-arachidonic acid which gave a ratio of 1:1 for the specific activities of the palmitoyl and the stearoyl species. This indicates that the arachidonic and the phosphoric acids do not enter the lecithin molecules at the same rate or by the same pathway. In accordance with our findings in vivo, Possmayer et al. (20) have demonstrated that arachidonic acid is much more readily introduced into the lecithin than into the phosphatidic acid by rat liver microsomes. If both arachidonoyl lecithins had arisen via acyl transfer a ratio of 1.35 of palmitoyl over stearoyl arachidonates would have been expected, as compared to the experimentally determined values of 0.82-1.27. The present results therefore suggest (31) that 1-stearoyl 2-arachidonoyl lecithin, and perhaps to a lesser extent the palmitoyl homologue were formed mainly via transfer of arachidonic acid to endogenous lysolecithin as claimed previously from in vitro studies (32).

It has also been speculated (27) that the arachidonoyl lecithins might be synthesized mainly by methylation of phosphatidyl ethanolamine. In apparent agreement with this concept is the finding from P^{32} studies (4) that the arachidonoyl cephalins possess a specific activity considerably greater than the corresponding lecithins. Since our studies with labeled acid gave arachidonoyl lecithins with a specific activity twice that of the corresponding cephalins, it would seem that arachidonic acid was not entering lecithin by methylation of cephalin as a major pathway. It is possible, however, that some palmitoyl arachidonoyl species could have been derived by preferential methylation of appropriate phosphatidyl ethanolamines (2,33).

In view of the sparsity of data available on the metabolism of the homologous phosphatidyl ethanolamines with which to compare our results obtained with labeled acids, it was not possible to deduce the mechanism for incorpo-

ration of the acids into the cephalins in vivo.

It must be pointed out that our data for each time period is based on one or two animals and a very small percentage of the administered radioactivity. The reutilization of acetate, from degradation of the labeled substrate, for the de novo synthesis and elongation of fatty acids could affect the data and the conclusions drawn.

The differential rates of incorporation of the linoleic and arachidonic acids into the palmitoyl and stearoyl lecithins and cephalins of rat liver emphasize the heterogeneity of the metabolism of glycerophosphatides, which had been previously demonstrated by means of labeled glycerol, phosphate and the nitrogenous bases. It is hoped that in the future it will be possible to interrelate the turnover of specific parts of the glycerophosphatide molecules to each other and to important molecular events in the metabolism of the cell and tissue.

ACKNOWLEDGMENTS

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Incorporation of P^{32} Orthophosphate Into Phospholipid of Epiphyseal Cartilage

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ABSTRACT

Injected P^{32} orthophosphate was found to be incorporated into phospholipids in the epiphyseal cartilage of young chicks. The phospholipid levels were found to be greater in the ossifying cartilage and in new bone then in the resting cartilage. Phosphatidyl choline was the most heavily labeled lipid, reaching peak specific activity at 24-26 hr. The specific activity of phosphatidyl ethanolamine continued to increase for the 48 hr period of the experiment. Phosphatidyl serine and sphingomyeline were labeled to a lesser extent; however phosphatidyl serine displayed the most rapid turnover of any of the phospholipid studied, attaining peak specific activity at 12-15 hr.

INTRODUCTION

Protein and polysaccharide have for many years been recognized to be the major constituent of the extracellular substances of epiphyseal cartilage. This has been shown, in recent works, to contain lipid too. Studies by Irving (1-4) and Melcher (5) have demonstrated the presence of lipid in epiphyseal cartilage at the site of active calcification where calcium was being laid down. The lipid stained with Sudan black B; Irving and Wuthier (6) suggested that it was phospholipid. Fels (7), Mikulecky and Tobias (8) and Irving and Wuthier (9) believed a binding of phospholipid occurred at these sites, in a protein-polysaccharide-phospholipid complex which was the active compound involved in bone calcification.

In an effort to define further the role of lipids, particularly phospholipids, in calcification, we studied phospholipid synthesis in rapidly calcifying epiphyseal cartilage from young chicks, in vivo.

EXPERIMENTAL PROCEDURE

White Leghorn chicks, 10-15 days old, were injected with 300 μc P^{32} orthophosphate/100 g body weight. P^{32} orthophosphate was obtained from New England Nuclear, Boston, Mass. Groups of six birds were decapitated every 4 hr over 48 hr, but over the period of the peak phosphorus incorporation, the intervals between samples were shorter.

Four to 10 min after death the proximal tibial epiphyses were removed from their shafts and cleaned of all soft tissues. They were then

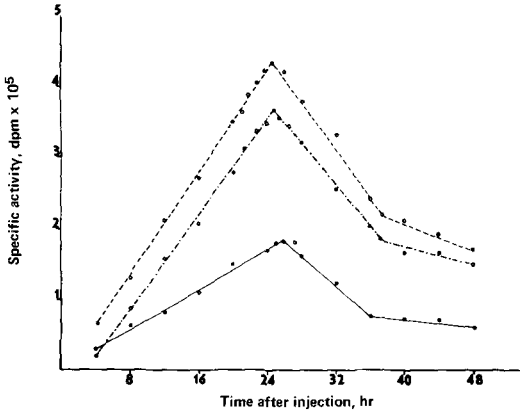

FIG. 1. Incorporation of P^{32} orthophosphate into phosphatidyl choline of epiphyseal cartilage. Three groups of six animals were injected P^{32} orthophosphate, each animal received 300 μc/100 g body weight, after time periods indicated, radioactivity of tibial epiphyseal cartilage was measured. Legend: ——— resting cartilage, –•–•– new bone, – – – ossifying cartilage.

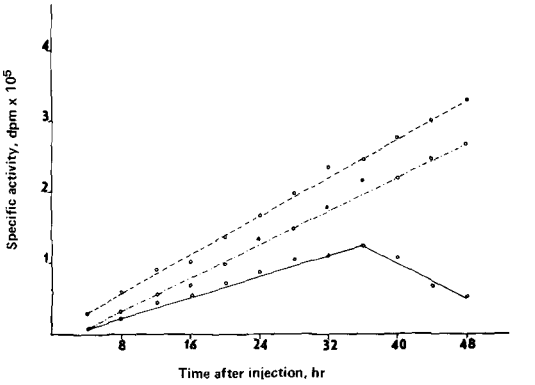

FIG. 2. Incorporation of P^{32} orthophosphate into phosphatidyl-ethanolamine of epiphyseal cartilage. Three groups of six animals were injected P^{32} orthophosphate, each animal received 300 μc/100 g body weight, after time period indicated, radioactivity of tibial epiphyseal cartilage was measured. Legend: ——— resting cartilage, –•–•– new bone, – – – ossifying cartilage.

TABLE I

Components of Chick Epiphyseal Cartilage[a]

Samples	Tissue weight, mg			Dry tissue, %	
	Wet	Dry	Ash	Total lipids	Phospho-lipids
Resting cartilage	595.8 ± 72[b]	134.1 ± 11.30	2.76 ± 0.51	2.77 ± 0.30	0.85 ± 0.13
Ossifying cartilage	270.3 ± 45	49.2 ± 6.50	1.62 ± 0.17	4.62 ± 0.73	2.32 ± 0.43
New bone	291.0 ± 54	81.6 ± 8.90	20.89 ± 1.90	4.82 ± 0.49	2.77 ± 0.51

[a]Samples were cleaned, weighed and dried at 105 C for 24 hr. The inorganic content was determined by ashing at 550 C for 24 hr. Values expressed as means of six groups each consisting of six animals.

[b]S.D.

divided up into resting cartilage, ossifying cartilage and new bone. Blood traces were removed by washing the slices with cold saline. The samples were homogenized with a "Virtis 45" at 4 C for 3 min.

The total lipids were extracted three times by the Folch (10) procedure. The tissues were then decalcified with 0.5 M EDTA for 48 hr, dialyzed and lyophilized. Then the lipid extraction was repeated another three times by the same method, and the tissue finally extracted with slightly acid methanol-chloroform (11); the acidic extract was neutralized. The extract containing the lipids was evaporated to dryness under a reduced pressure and purified using a sephadex column (12). The total lipids were determined gravimetrically. The phospholipids were separated on silicic acid columns, neutral lipid being eluted with chloroform and phospholipids with methanol.

Identification and separation was carried out by two-dimensional chromatography on silica gel loaded paper (11). The various lipids were detected on chromatograms by staining the dried papers with 0.001% aqueous solution rhodamine 6G and viewing under UV light (3660 A). Autoradiograms were developed on "Gevaert Osray M. Blue base" film. The radioactive areas were cut out, and eluted with methanol and dissolved in 10 ml of counting solution (0.4% PPO; 0.005% POPOP per liter toluene). The recovery of the radioactivity from the chromatograms was 78-90%. No residual organic phosphorus could have been detected following ashing of the chromatograms after the elution. The samples were counted in a Packard Tri Carb Liquid scintillation spectrometer (Model 3380). The degree of quenching was estimated by the Channel ratio method and the data suitably corrected. Specific activity was expressed in terms of dpm/mg phospholipid. The phospholipids were assayed by measuring their phosphorus content (13).

RESULTS

In Table I the values of wet and dry weight and ash of epiphyseal cartilage are given. Mois-

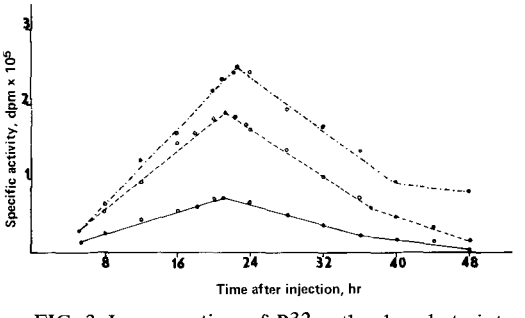

FIG. 3. Incorporation of P^{32} orthophosphate into sphingomyelin. Three groups of six animals were injected P^{32} orthophosphate 300 μc/100 g body weight, for varying time periods. New bone was the most heavily labeled at all time period reach a peak at 22 hr. Legend: ——— resting cartilage, —•—•— new bone, — — — ossifying cartilage.

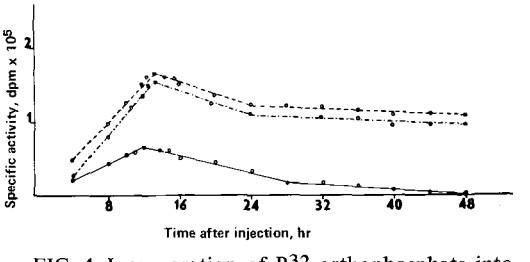

FIG. 4. Incorporation of P^{32} orthophosphate into phosphatidyl-serine. Three groups of six animals were injected P^{32} orthophosphate 300 μc/100 g body weight, for varying time periods. Ossifying cartilage and new bone reach a peak after 15 hr. Legend: ——— resting cartilage, —•—•— new bone, — — — ossifying cartilage.

TABLE II

Phospholipid Composition of Epiphyseal Cartilage[a]

Phospholipid	Total lipids P[b], %		
	Cartilage		
	Resting	Ossifying	New bone
Sphingomyelin	6.4 ± 0.07[c]	6.18 ± 0.17	5.3 ± 0.30
Phosphatidylcholine[d]	61.2 ± 0.71	53.8 ± 0.36	46.8 ± 1.00
Phosphatidylethanolamine[d]	14.7 ± 0.28	23.2 ± 0.52	24.1 ± 0.52
Phosphatidyl serine[d]	5.4 ± 0.21	4.5 ± 0.01	5.2 ± 0.23
Phosphatidyl inositol	4.4 ± 0.21	3.8 ± 0.13	3.2 ± 0.18
Phosphatidic acid	0.9 ± 0.02	3.4 ± 0.09	0.9 ± 0.04
Total P μg	153-340	520-900	740-1100

[a]The various phospholipid classes were separated on silica gel loaded paper; 5-10 μg lipid phosphorus was used per chromatogram. The chromatograms were developed by ascending chromatography in the first direction using chloroform-methanol-2,6 dimethyl 4 heptanone-acetic acid-water (45:15:30:20:4, v/v) and allowed to dry. In the second direction they were developed with chloroform-methanol-2,6, dimethyl 4 heptanone-pyridine-0.5 M pH 10.4 ammonium chloride buffer (30:25:25:35:8, v/v).

[b]Percentage of the total lipid P was obtained from the silica gel-loaded paper chromatogram. The recovery of P was 91-96%.

[c]Mean of 46-50 samples ± SEM.

[d]These phospholipids included the lysophospholipids.

ture content of cartilage was 77-80%. Ash content of cartilage was about 2-3% that of new bone, 21%. The total lipids extracted from various epiphyseal zones are shown in Table I. As will be seen, the lipid content of the cartilage increased as mineralization progressed. The total lipid content was greatest in the ossifying cartilage and in the new bone. The highest phospholipid level was found in new bone.

Table II shows the phospholipid composition of the epiphyseal cartilage. A large amount of phosphatidyl choline was found in all zones. In the ossifying cartilage, phosphatidyl ethanolamine was present in a higher proportion than in the resting cartilage.

Figures 1, 2, 3 and 4 present the incorporation of injected P^{32} orthophosphate into phospholipid. As can be seen, the radioactivity of all phospholipids increased with time and reached a peak value between 15 and 36 hr. An exception was phosphatidyl ethanolamine which increased in the ossifying and new bone until the end of the experiment at 48 hr. Resting cartilage showed consistently lower labeling than the other zone.

Phosphatidyl choline was the most heavily labeled phospholipid. At the peak point 50-60% of the total P^{32} orthophosphate incorporated was present in phosphatidyl choline (Fig. 1).

Phosphatidyl ethanolamine contained 40-50% of the P^{32} orthophosphate incorporated at 48 hr, the other phospholipids decreasing in P^{32} label at this time (Fig. 2).

Sphingomyelin (Fig. 3) reached a peak between 21-23 hr. A small amount of phosphatidyl serine was detected on the chromatogram (Table II). Its maximum specific activity was found at 12-15 hr, the earliest peak of any of the phospholipids studied (Fig. 4). Three additional radioactive phospholipids were detected on the chromatograms. One gave a positive inositol test with ammoniacal silver nitrate. The second was phosphatidic acid and the third diphosphatidyl glycerol. In these phospholipids the amount of P^{32} orthophosphate incorporated was low.

DISCUSSION

These studies revealed that injected radioactive phosphate is readily incorporated into chick epiphyseal phospholipids, confirming earlier in vitro studies with other precursors that ossifying tissues have active metabolism (14-19). The major route for the biosynthesis of phosphatidyl choline has been shown to occur via the diglyceride-CDP-choline pathway in most mammalian tissue studied (20) and also in bones and bone cell cultures (19). Orthophosphate contributes to the labeling of phosphatidyl choline through CDP-choline and to phosphatidyl ethanolamine through CDP-ethanolamine, whereas the phosphatidyl inositol may be labeled via glycerol phosphate through the reaction of CDP diglyceride + inositol (20). Dirksen et al. reported that sphingomyelin and phosphatidyl serine were not labeled in calvaria bone incubated with P^{32} orthophosphate but were incorporated after 4 hr incubation in a

medium containing C^{14} serine.

It is known (21) that there are metabolic differences between the zones of epiphyseal cartilage and the newly formed bone. The levels of phospholipid in the ossifying region and in the new bone were found to be more or less equal. Because of the high specific activity of the phospholipid in these two zones and the close relationship between the extractability of the acidic phospholipids and the level of mineral a direct interaction between these lipids and bone minerals has been suggested (22-25). Having found phospholipids at the site of calcification the question arises as to whether acidic lipids are directly involved in the calcification process (9). The possibility exists that phospholipids may be utilized or bound during calcification. This fits in with similar findings on bovine fetal cartilage by Wuthier and Irving et al. (9,22), who showed that ossifying cartilage is far richer in phospholipid than are the adjacent zones.

The high level of incorporation of P^{32} orthophosphate into ossifying cartilage and new bone permit several suggestions as to how phospholipids are involved in bone formation. Termine et al. (26) suggest that a complex of protein-polysaccharide phospholipids and amorphous calcium phosphate may be formed during calcification. Phospholipids may serve as agents for cation transfer (e.g. for Ca^{++}) to the calcifying areas (9,22). The well-known affinity of phospholipids for calcium and their ability to form membrane-like structures have been emphasized by Johnson (27).

The increase of phospholipid synthesis in the ossifying cartilage and in new bone strongly supports the first two theories on the relationship between phospholipid and bone formation.

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Distribution and Metabolism of Two Orally Administered Esters of Tocopherol[1]

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ABSTRACT

A comparison of the distribution of total radioactivity in rat tissue lipids after the oral administration of d,l-3,4-$^3H_2$-α-tocopheryl nicotinate and d,l-α-tocopheryl-1',2'-$^3H_2$-acetate in equimolar concentrations has demonstrated that there is considerable variation in the concentration of vitamin E in organs at different times after dosing. A higher total radioactivity was found in the tissues of animals receiving α-tocopheryl nicotinate than after α-tocopheryl acetate 12 hr after feeding with an emulsion, but not at most other time intervals studied. These findings indicate that the tissue uptake of vitamin E after oral dosage with nicotinate ester is, perhaps, poorer than that occurring after feeding with tocopheryl acetate, or that α-tocopheryl nicotinate has a faster turnover than the acetate ester. Although total radioactivity in the blood and liver of those animals dosed with α-tocopheryl acetate varied slightly with time, there was a high peak of radioactivity at 12 hr after dosage with nicotinate ester. In both groups of rats, the adrenals, ovaries, adipose tissue and heart appeared to extract vitamin E from the blood for a period of up to 48 hr postabsorptively. Metabolic products of tocopherol detected by glass-fiber paper chromatography were found in both instances. This analysis revealed that, when orally administered, both α-tocopheryl nicotinate and α-tocopheryl acetate are extensively metabolized by the tissues of the rat. The metabolite most abundantly occurring under these conditions was α-tocopheryl quinone. In the adrenal glands, however, the most highly labeled compound was unesterified tocopherol, which increased with time and comprised up to 90% of the chromatographed radioactivity. From the data obtained, it can be assumed that the adrenal tissue plays a definite role in the metabolism of vitamin E.

INTRODUCTION

Evidence has recently been presented (1,2) indicating that a higher concentration of tocopherol appears in the lymph when α-tocopheryl nicotinate is administered to rats with a thoracic duct fistula than when α-tocopheryl acetate is given. This difference in absorption and lymphatic transport could result in higher tissue concentrations of vitamin E following oral dosage with the nicotinate ester. Because it is possible that some of the suspected biological activities of vitamin E (such as its possible "anti-inflammatory action") can take place only when the vitamin is present in adequately high concentrations at its site of action (target tissue), we have engaged in the search of vitamin E derivatives capable of producing high tissue concentrations of this vitamin.

The object of the present investigation was to determine the organ distribution, storage and metabolism of orally administered α-tocopheryl nicotinate, and to correlate these findings with those obtained after the administration of α-tocopheryl acetate. The latter was selected for comparison purposes because it constitutes the most widely used form of vitamin E in animals and humans.

EXPERIMENTAL PROCEDURES

Female albino rats (CD Charles River Breeding Labs., North Wilmington, Mass.), weighing between 230 and 250 g, were maintained on a diet of laboratory Purina chow and water, ad lib. The animals were fasted overnight before administration by stomach tubes of an emulsion containing labeled vitamin E ester. The emulsion (4 ml) was of the same composition as that utilized in studies of cholesterol absorption and has been described in detail elsewhere (3). Ethanolic solutions containing 2.3 mg of d,l-α-tocopheryl nicotinate mixed with 50 μC of d,l-3,4$^3H_2$-α-tocopheryl nicotinate were added to the emulsion and administered to a group of rats. Another group of animals received the same emulsion in which had been dissolved ethanolic solutions of 2 mg

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of d,l-α-tocopheryl acetate and 50 μC of d,l-α-tocopheryl-1',2'-$^3H_2$-acetate. In both instances the test emulsion was given with the aid of a steel oral cannula to unanesthesized animals. The rats were subsequently confined to restraining cages to prevent coprophagy, and were allowed to drink .85% saline ad lib. during the entire duration of the experiment. Both the radioactive substances and the vitamin E carrier esters were products synthesized at Roche Laboratories. When checked for purity by either thin layer silicic acid or glass-fiber paper chromatography using liquid scintillation counting (1-3), the radioactive substances were found to be > 99% pure; these compounds were, therefore, used without further purification.

Collection of Samples

The animals were subdivided into groups of two, taken out of the restraining cages, anesthesized with Penthrane (Methoxyfluorane, Abbott Laboratory, North Chicago, Ill.) and killed 3, 6, 12, 24 or 48 hr after administration of the emulsion. As much blood as possible was taken from the abdominal aorta. The samples of blood were collected with heparinized tubes. A sample of skeletal muscle was taken from both thighs and adipose tissue was removed from the latus and epididimal fat pads. The visceral organs were removed immediately after taking the blood sample, rinsed two or three times with distilled water, weighed and kept frozen until further processing.

Tissue Lipid Extraction and Radioactive Measurements

Samples of total blood were extracted and processed as described for cholesterol absorption studies (3). The extracting solvents were purified as reported previously (3). The heart, skeletal muscle, liver and kidney were homogenized with a minimum amount of water in a drill press (Sears, Roebuck and Co.) at 1000 rpm. After complete homogenization was achieved, the organs were lyophilized in a Virtis Freeze-Drying Unit and the lipids were subsequently extracted with varying amounts of ethanol-isopropyl ether (2:1).

The spleen, adrenals, ovaries and adipose tissue were homogenized vigorously and extracted simultaneously with ethanol-isopropyl ether, using 30 ml extracting solvent per g of wet tissue.

After extraction, the samples were centrifuged at 2000 rpm, the supernatant phase removed, evaporated to dryness under N_2, and finally dissolved in 0.4 to 4 ml volumes of extracting mixture. To measure total radioactivity, one aliquot was applied to a small piece of glass-fiber paper and allowed to dry. The radioactivity was then counted. Another aliquot was utilized to separate the tocopherol and its metabolites from the lipids. This was done by glass-fiber paper chromatography (ITLC-SG, Gelman Instruments Co., Ann Arbor, Mich.). Solvent systems recently developed for the separation of vitamin E metabolites in lymph (2) were used. In all instances, measurement of radioactivity was achieved by using a Packard Tri-Carb liquid scintillation Spectrometer, with Model 544 attachment (1). The results are thus reported in dpm/g wet tissue and there was no significant quenching by the samples used.

RESULTS AND DISCUSSION

Figure 1 illustrates the appearance of total radioactive vitamin E in the blood, liver, spleen and kidney of rats orally dosed with either α-tocopheryl nicotinate or α-tocopheryl acetate. The uptake of radioactivity resulting from administered nicotinate ester was higher than that from acetate ester feeding only 12 hr after administration of the emulsion. At most other experimental periods a higher uptake of vitamin E after dosage with α-tocopheryl acetate was observed than after α-tocopheryl nicotinate. After dosage with α-tocopheryl acetate the labeled vitamin E in the blood increased slightly with time. This concentration of radiovitamin E in the blood is the result of a balance between the rates of absorption from the gastrointestinal tract, release by the liver and the concomitant metabolism by peripheral tissues.

Both α-tocopheryl nicotinate and α-tocopheryl acetate were readily converted in the studied animals to free tocopherol and its metabolites (see Table I). This conversion presumably started at the level of the intestinal mucosa (1,2). After dosage with either of the two tocopherol esters, most of the radioactivity was recovered as tocopheryl quinone. Under the present experimental conditions, there was a significantly higher concentration of free tocopherol in the blood after the administration of vitamin E acetate than after nicotinate, thus indicating that dosage with α-tocopheryl acetate is of greater effect than α-tocopheryl nicotinate as regards α-tocopherol in the blood. It is possible that these differences in concentration in the blood tocopherol of the two groups of animals may correlate with differences in the biological activity of these two tocopherol esters.

It was previously shown that independently of the nature of the administered tocopherol

TABLE I

The Appearance of Tocopherol and its Metabolites in Tissues of Rats 12 and 48 hr After the Oral Administration of *d,l*-3,4-$^3H_2$-α-Tocopheryl Nicotinate or *d,l*-α-Tocopheryl-1',2'-$^3H_2$-Acetate

	Blood		Liver		Spleen		Kidney		Adrenals		Heart		Ovaries		Adipose tissue		Skeletal muscle	
	12	48	12	48	12	48	12	48	12	48	12	48	12	48	12	48	12	48
Tocopheryl nicotinate[b]	8[c]	8	8	11	5	6	7	5	1	1	6	5	13	6	8	9	4	6
Tocopherol	13	3	4	4	61	35	29	21	74	90	14	38	23	34	31	28	58	11
Tocopherol dimer[d]	7	7	1	1	0	0	0	0	0	0	0	0	0	0	3	2	0	0
Tocopheryl quinone	62	75	84	78	33	54	62	71	25	9	61	49	58	56	51	55	34	79
Unidentified oxidation products	10	7	3	6	1	5	2	3	0	0	19	8	6	4	7	6	4	4
Tocopheryl acetate	2	1	1	1	2	2	1	0	0	0	1	1	1	0	3	3	1	0
Tocopherol	22	17	5	4	37	37	24	20	79	82	13	26	17	35	27	27	40	66
Tocopherol dimer	3	1	1	3	0	0	1	0	0	0	0	0	0	0	3	1	0	0
Tocopheryl quinone	60	73	86	83	54	52	70	67	21	18	65	58	67	59	47	60	54	28
Unidentified oxidation products	13	8	7	9	7	9	4	13	0	0	21	15	15	6	29	9	5	6

[a]Experimental procedure as described in Figure 1. The lipid extracts were chromatographed on glass-fiber paper and the two solvent systems recently described (2) were used.

[b]The tocopherol esters as well as the other reference compounds (free tocopherol, dimer and tocopheryl quinone) were all products synthesized at Roche Laboratories.

[c]Per cent of chromatographed radioactivity (rounded figures).

[d]This represents ^3H-radioactivity in the chromatographic region corresponding to tocopherol dimer.

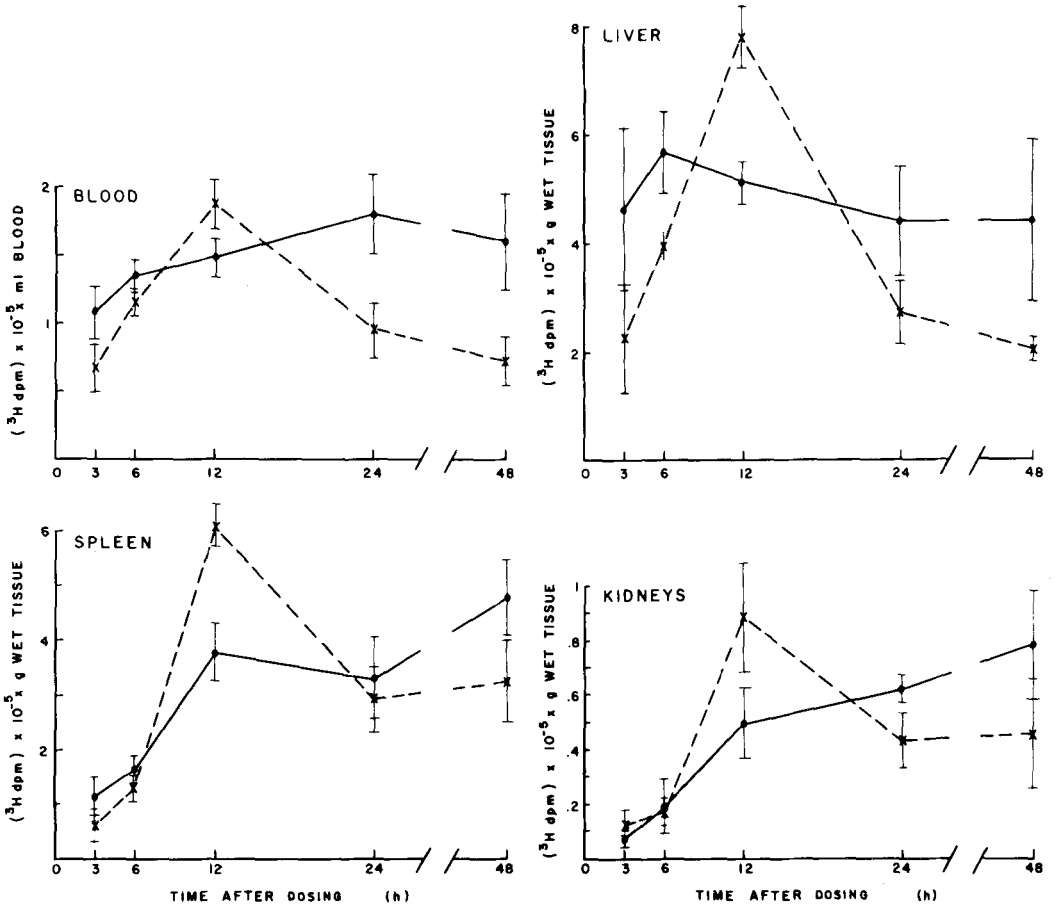

FIG. 1. The appearance of total ^{3}H-radioactivity in the blood, liver, spleen and kidneys of rats, after the oral administration of d,l-3,4-$^3H_2$-α-tocopheryl nicotinate (x------x) or d,l-α-tocopheryl-1′,2′-$^3H_2$-acetate (•————•) to normal rats. Four ml of an emulsion containing protein, carbohydrate, monoolein, saline and the radioactive vitamin E ester were given by stomach tube. The animals were killed at specified time intervals and the total radioactivity in the organs was analyzed as described in the text.

ester, the bulk of the radioactivity appearing in the lymph of rats is associated with free tocopherol (1,2). The present experiments show that, regardless of the ester of tocopherol given, most of the radioactivity recovered in the blood, liver and other organs, is associated with tocopheryl-*p*-quinone. The reason for the difference in composition of vitamin E metabolites in the lymph and other tissues of rats on a normal diet is not yet clear. It may be pertinent to mention that although after dosage with tocopheryl nicotinate the lymph contained predominantly free tocopherol, a small percentage of radioactivity appearing in the liver of these lymph-fistulated animals (2) was associated almost entirely with tocopheryl quinone.

Only traces of the acetate ester, as such, appeared in the blood of animals dosed with α-tocopheryl acetate, although higher amounts of the nicotinate ester per se were detected when α-tocopheryl nicotinate was administered. These findings were expected in view of our recent reports (1,2) on the lymphatic appearance of these two compounds. A higher amount of the intragastrically administered nicotinate escaped hydrolysis than that of the acetate. These observed results may be related to differences in the specificity and site of action of the lipolytic pancreatic enzymes acting on these two substrates. Complete hydrolysis of α-tocopheryl acetate would occur in the intestinal lumen prior to passage through the mucosal wall. Deesterification of α-tocopheryl nicotinate, on the other hand, may not be complete, thus allowing some of this ester to appear as such in the lymph and, subsequently, in the blood, liver and other tissues, as demonstrated in Table I.

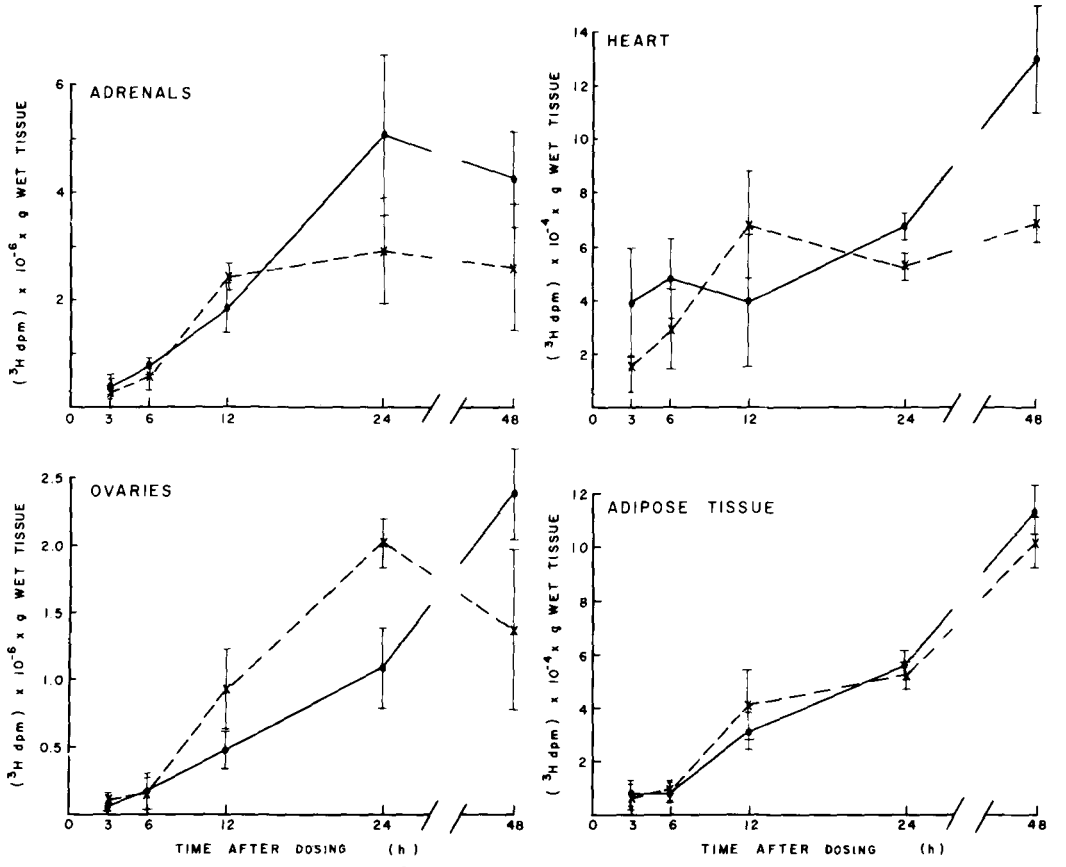

FIG. 2. The appearance of total ^{3}H-radioactivity in the adrenals, heart, ovaries and adipose tissue of normal rats dosed orally with two esters of tocopherol. Experimental procedure and other explanations as described in Figure 1.

The above findings in the rat do not agree with the observations that, following its oral administration, α-tocopheryl nicotinate appears primarily as the fed ester in the blood and liver of vitamin E-deficient chickens (R. Bunnel, personal communication). At the moment, these findings are difficult to reconcile, although they may simply represent species differences in the intestinal absorption of the nicotinate ester.

When the total radioactivity in the liver was analyzed, it was found that it contained the highest concentration of radiovitamin E in both groups of experimental animals. Although difficult to eliminate in this type of experiment, contamination with blood probably played no significant role in producing the high concentration of radioactivity found in the liver of these animals, because there were marked qualitative and quantitative differences between these two tissues during all experimental periods. The liver is, therefore, the main site of storage of vitamin E. Although radioactivity concentration, as assessed by dpm/g wet tissue, showed only small variations with time in the group of rats dosed with α-tocopheryl acetate, in animals dosed with α-tocopheryl nicotinate a significant time course variation was observed, maximum storage occurring 12 hr after administration of this ester, after which the concentration declined to the levels found in the early postabsorptive periods (Fig. 1). These findings reflect differences in the rate of absorption of these two vitamin E esters and also point out the important role of the liver in the regulation of vitamin E dynamics of pool sizes.

Analysis of the hepatic metabolites by glass-fiber paper chromatographic methods revealed that most of the radioactivity was as α-tocopheryl quinone (4,5). It was found that (Table I) up to 84% of the chromatographed radioactivity in the animals given α-tocopheryl nicotinate and up to 86% in those animals fed α-tocopheryl acetate was associated with the quinone. The observed high concentration of

tocopheryl quinone in the liver is at variance with the report of other investigators (6,7) who were unable to show a significant accumulation of the quinone in the livers of rats or chickens dosed with free ^{14}C-α-tocopherol.

The reasons for the above discrepancies are not clear, but it might be worth mentioning that the experimental conditions used by other investigators were very different from ours. We used normal rats and fed the tocopherol ester in a relatively large volume (4 ml) of an emulsion containing protein, carbohydrate and a monoglyceride. Thus, the method of administration of the vitamin, whether in a free form or esterified, the vehicle (aqueous, emulsion), the size of the dosage, the species and nutritional status of the animal, etc., are all factors which have to be considered when trying to explain such discrepancies. In addition, differences in the technical procedure employed should also be considered, because α-tocopheryl quinone, a product of chromanol oxidation, can arise as an artifact during isolation procedures (8). This possibility was ruled out in the present experiments because the adrenal tissue showed low concentrations of tocopheryl quinone in the two groups of animals. Samples of tissue containing high amounts of lipids were treated with silicic acid, and after this procedure the content of α-tocopheryl quinone did not vary in the sample. Moreover, rats dosed intravenously with either nicotinate or acetate ester (Gallo-Torres and Miller, unpublished data) showed only a very minor conversion of the ester to quinone in their livers, although the analytical procedure utilized was the same as the one reported here. This rules out the possibility of the quinone being an artifact during extraction procedures.

The rate of appearance of radioactivity in the spleen and kidney is also shown in Figure 1. Radiovitamin E increased steadily with time in the rats to which α-tocopheryl acetate was given, but less markedly in those dosed with α-tocopheryl nicotinate. In the latter group a high peak of radioactivity was seen in both tissues at the above-mentioned 12 hr period. A study of the splenic metabolites revealed that only 12 hr after feeding there was a higher appearance of unesterified tocopherol in the animals receiving the nicotinate ester as judged by per cent of chromatographed radioactivity (Table I). The analysis of the metabolic products in the kidney revealed that tocopheryl quinone accounted for most of the chromatographed radioactivity. No qualitative differences were observed in the two groups of rats as regards renal metabolites.

The data in Figure 2 depict the uptake of

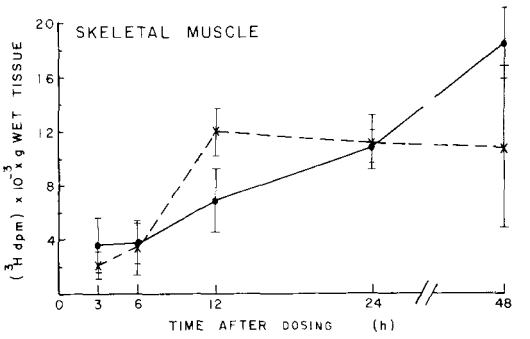

FIG. 3. The appearance of total ^{3}H-radioactivity in the skeletal muscle of normal rats dosed orally with two esters of tocopherol. Experimental procedure and other explanations as described in Figure 1.

radioactivity by the adrenals, heart, ovaries and adipose tissue of animals dosed with either vitamin E nicotinate or acetate. When expressed in dpm/g wet tissue, the adrenal gland showed the greatest accumulation of radioactivity. A significant finding was the demonstration of a gradual and linear increase in the adrenal radioactivity when the animals received α-tocopheryl acetate, although the uptake in the animals dosed with α-tocopheryl nicotinate was significantly lower. In both groups, however, an increasing ratio of adrenal-blood radioactivity was seen. Thus, 3, 6, 12, 24 and 48 hr after administration of the emulsion, the corresponding ratio was 3, 6, 12, 32 and 32. This increment with time in the adrenal-blood radioactivity ratio was observed in animals dosed with α-tocopheryl nicotinate as well as in those receiving α-tocopheryl acetate. These findings are in accord with the observation of Weber et al. (9) who showed that 24 hr after the oral administration of l-α- or d-α-tocopherol to rats, the concentration of these two isomers in the adrenal gland was respectively 32 and 37 times higher than in the blood.

Further indication of the important role played by the adrenal gland in the metabolism of vitamin E was obtained with the analysis of the labeled metabolites in that organ. Regardless of the ester fed, the most abundant compound appearing in the adrenal was free tocopherol which increased with time (Table I). Thus, in animals dosed respectively with vitamin E acetate or nicotinate, 3 hr after feeding 75% and 67% of the chromatographed radioactivity from the samples was associated with unesterified tocopherol. This proportion of free tocopherol was even higher 48 hr postprandially, when the corresponding fractions reached as much as 82% and 90%. None of the other tissues examined showed such a high concentration of α-tocopherol. These results sug-

gest that the adrenal gland plays some as yet undefined role in the metabolism of vitamin E. More information is required on the effect of α-tocopherol on the adrenal function and its interrelationship with other vitamins (notably ascorbic acid, vitamin A and pyridoxine) suspected to have an effect on this organ. Thus, the demonstration that the adrenal gland also accumulates a considerable amount of vitamin C should be further evaluated in experiments in which vitamin E is concomitantly studied, since it has been demonstrated in vitro that ascorbic acid and tocopherol potentiate in their antioxidant effect (10). One of the actions of vitamin E is to limit lipid peroxidation. Therefore, a minimum of peroxidation of the adrenal lipids can be expected in cases when the concentration of these two vitamins has reached an adequate level. This, however, remains to be properly documented.

The rapidly metabolizing myocardial tissue showed an increasing uptake of radioactivity (Fig. 2) during the first 48 hr after feeding rats on a normal diet with α-tocopheryl acetate or α-tocopheryl nicotinate. Glass-fiber paper chromatography of the heart lipids revealed a very high concentration of oxidation products which remained at the origin of the chromatograms and were more polar than tocopheryl quinone and α-tocopheryl nicotinate (Table I). The nature of these more polar vitamin E compounds remains to be clarified, and also whether their existence is related to the metabolic activity of the cardiac muscle.

There was an increase with time in the radiovitamin E in the ovaries of the two groups of animals. Up to 24 hr after dosage, the increase in uptake by ovarian tissue was far greater and more obvious in the nicotinate group than in the acetate group. However, at 48 hr the acetate group uptake surpassed that of the nicotinate group. Most of the radioactivity recovered in this tissue was also associated with α-tocopheryl quinone.

Figure 2 also shows that there was no significant difference in the uptake of total radioactivity by the adipose tissue after administration of either tocopherol ester. The uptake of radioactivities by adipocytes at 48 hr indicates the importance of this tissue in vitamin E storage. Assuming a normal distribution of fat, this could account for 30% of the body store. The observed parallelism after feeding with either tocopherol ester was not only quantitative but also qualitative, since most of the chromatographed radioactivity was associated with tocopheryl quinone in both instances. Here, it is pertinent to mention that Weber and Wiss (11) reported that certain dietary polyunsaturated fatty acids produced a considerable increase of α-tocopheryl quinone in the depot of rats. In their studies, as well as in the present report, the level of unchanged α-tocopherol was found to be simultaneously decreased.

As shown in Figure 3, there was an increase in radioactivity with time in animals dosed with either tocopheryl nicotinate or tocopheryl acetate. Twelve hours after dosage, the value for the group dosed with nicotinate was larger than that for the group dosed with acetate. However, at 48 hr the reverse was found. The relative proportion of vitamin E metabolites was different in both groups of rats (Table I). Thus, in animals receiving vitamin E nicotinate, the tocopheryl quinone was about 34% of the chromatographed radioactivity 3 hr postprandially, but at 48 hr it had increased to 79%. On the other hand, 3 hr after dosage with vitamin E acetate, tocopheryl quinone constituted 54% of the chromatographed radioactivity which decreased to lower concentrations (28%). Concomitantly, 48 hr after administration of α-tocopheryl acetate, most of the chromatographed radioactivity (66%) was associated with free tocopherol, but this was not the case when α-tocopheryl nicotinate was fed. These dissimilarities reflect a marked difference in the metabolism of the two compounds in muscular tissue.

When the nicotinate ester was administered, a low, but definite, percentage of the chromatographed radioactivity found in the examined tissues was associated with α-tocopheryl nicotinate, as such. In contrast, after administration of the acetate ester, only traces of α-tocopheryl acetate appeared in the tissues. In addition, regardless of the time interval studied, most of the tissues showed a significantly higher concentration of polar compounds after dosage with α-tocopheryl acetate than with α-tocopheryl nicotinate. These findings clearly emphasize that these two esters of tocopherol are metabolized to a different extent by the tissues of the rat.

Finally, chromatographed radioactivity corresponding to tocopherol dimer was either low or absent in the present experiments. This is evident from Table I. Three hours postabsorptively, the dimer appeared to be, at the most, 7% of the chromatographed radioactivity in the blood of animals given vitamin E nicotinate. An even lower concentration of this metabolite was seen in the blood of rats administered vitamin E acetate. The liver contained a smaller percentage of dimer than the blood at the corresponding observation periods. Some tissues contained only traces of it and others were virtually devoid of this metabolite. The

finding of variable concentrations of tocopherol dimer in the tissues of the rat is at variance with that of Strauch et al. (8) who were unable to demonstrate dimers after the oral administration of α-tocopherol to rats. These differences in findings are, perhaps, the result of different rates of oxidation of vitamin E in the different tissues examined.

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Characteristics of Fatty Acid Esterification by Homogenates of Bovine Mammary Tissue[1,2]

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ABSTRACT

Fatty acid esterifying activity of homogenates of bovine mammary tissue was associated with the particulate fraction of the cell, was strongly dependent upon ATP, CoA, D,L-glycerol-3-phosphate, and Mg^{2+}, and was stimulated by NaF, dithiothreitol and bovine serum albumin. The system made phospholipids, mono-, di- and triglycerides but did not esterify butyrate. The inability to form greater than 58% triglyceride suggested some factor(s) was limiting the acylation of di- to triglyceride. The results were consistent with glyceride synthesis by the α-glycerophosphate pathway.

INTRODUCTION

Accelerated lipid metabolism at parturition, synthesis and esterification of short chain fatty acids, and the synthesis of a product containing >98% triglycerides make the mammary gland an ideal tissue for investigating the regulation of glyceride synthesis (1). Fatty acid esterification by mammary tissue has been studied with cell free preparations from rats (2), guinea pigs (3-6) and goats (7,8). Although glyceride synthesis has been investigated in bovine mammary tissue slices (9) and dispersed cells (10-13) the specific requirements for glyceride synthesis by the bovine mammary gland are not known (1).

These studies devise a method of assaying glyceride synthesis (EC 6.2.1.3, EC 2.3.1.15, EC 3.1.3.4, EC 2.3.1.20) in homogenates of bovine mammary gland and partially characterize this fatty acid esterification.

[1]This work is part of a Ph.D. Thesis submitted by E.W. Askew to the Dairy Department and Institute of Nutrition, Michigan State University.

[2]Published with approval of the Director of the Michigan Agricultural Experiment Station as Journal Article No. 5101.

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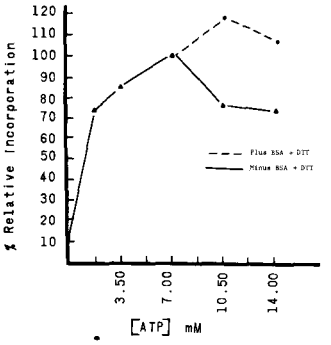

FIG. 1. Relative esterification of palmitate at five concentrations of ATP in the incubation mixture. Palmitate esterification at 7.0 mM ATP is represented as 100% to allow comparison of esterification rates in two separate experiments conducted in the presence (broken line) and absence (dark line) of BSA and DTT. Cofactor concentrations for the minus BSA + DTT incubations were as indicated in Table I. Cofactor concentrations for the plus BSA + DTT values were as above plus 5.0 mg BSA and 4.0 mM DTT.

MATERIALS AND METHODS

The procedures used were a modification of those given by McBride and Korn (4). Mammary tissue from 15 cows was used, but direct treatment comparisons were conducted simultaneously on the same tissue homogenate. Most of the values reported are averages of duplicate incubations. Mammary tissue from lactating cows was rinsed in ice cold 0.15 M KCl, blotted on cheese cloth and frozen immediately on dry ice. Thin slices of frozen tissue were disrupted in 8 vol of ice cold 0.15 M KCl with an Omni-Mixer (10,000 rev./min, no load) for 30 sec and then homogenized in a glass Teflon homogenizer (1000 rev./min). The homogenate was centrifuged 800 x *g* for 10 min at 0 C and the

TABLE I

In Vitro Assay System for Bovine Mammary Glyceride Synthesis[a]

Component	Concentration
ATP	10.5 mM
CoA	0.4 mM
D,L-glycerol-3-phosphate	20.0 mM
$HgCl_2$	2.0 mM
NaF	50.0 mM
Dithiothreitol (DTT)	4.0 mM
BSA	2.5 mg/ml
(1-^{14}C) palmitate	0.2 mM
800 x *g* supernatant	Variable
Phosphate buffer (pH 7.5)	90.0 mM

[a]Reaction mixture was incubated at pH 7.2 in a 2.0 ml volume at 37 C for 1 hr.

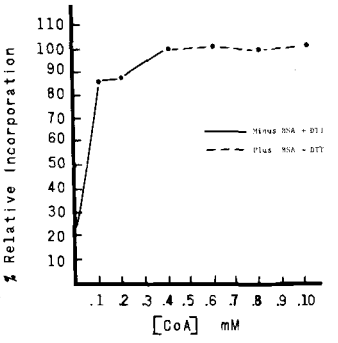

FIG. 2. Relative esterification of palmitate at six concentrations of CoA in the incubation mixture. Palmitate esterification at 0.4 mM CoA is represented at 100% to allow comparison of esterification rates in two separate experiments conducted in the presence (broken line) and absence (dark line) of BSA and DTT. Cofactor concentrations for the minus BSA + DTT incubations were: ATP (7.0 mM), CoA (as indicated), D,L-glycerol-3-phosphate (20.0 mM), $MgCl_2$ (2.0 mM), NaF (50.0 mM). Cofactor concentrations for the plus BSA + DTT system were as above plus 5.0 mg BSA and 4.0 mM DTT.

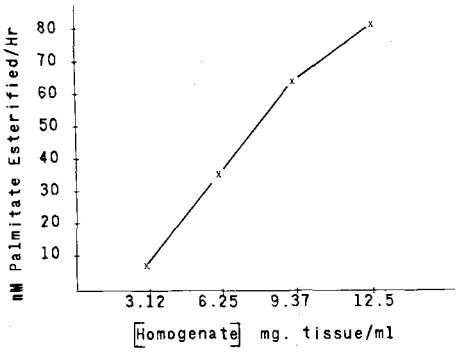

FIG. 3. Palmitate esterification in response to increasing concentrations of homogenate in the incubation mixture. Conditions of assay were those shown in Table I except concentration of homogenate (800 x *g* supernatant) was varied as indicated. Homogenate concentration is expressed as the milligram of tissue from which the homogenate was derived per milligram of incubation mixture. Tissues averaged approximately 75 mg extractable protein per gram. Similar results were obtained with two other tissue sources.

resulting supernatant filtered through glass wool. Further centrifugations were conducted as described by Pynadath and Kumar (8).

The 1-^{14}C-palmitic acid (99% chromatographically pure) was obtained from Nuclear-Chicago, Des Plaines, Ill., and (3,4-^{14}C) β-hydroxybutyric acid was a gift from C.L. Davis and D.S. Sachan, Department of Dairy Science, University of Illinois. Bovine serum albumin (fraction V), CoA, D,L-glycerol-3-phosphate, and ATP were from Sigma Chemical Co., St. Louis, Mo. Dithiothreitol was from Nutritional Biochemicals Corp., Cleveland, Ohio. Solvents were analytical reagent grade.

Approximately 2 μc of (^{14}C) fatty acid were added to 100 μmoles of its unlabeled analog and 2 ml of 0.1 N NaOH were added. The mixture was emulsified in an ultrasonic cleaner for 5 min. Three milliliters of 0.1 M phosphate buffer (pH 7.5) was added to bring the final volume to 5 ml. A typical substrate had a specific activity of 50,000 dpm/μmole fatty acid. Sonication of the substrate immediately prior to assay yielded an emulsion that could be aliquoted accurately.

The optimum mixture found for the assay of glyceride synthesis is shown in Table I. The reactants in a final volume of 2.0 ml at pH 7.2 were incubated in 25 ml glass stoppered flasks at 37 C for 1 hr with gentle shaking. The reaction was terminated by adding 8.0 ml heptane-isopropanol (1:1 v/v) and 6.0 ml of 0.03 M NaOH. The mixture was transferred into a test tube and allowed to separate into two layers. The upper layer was washed twice with 6.0 ml of 0.03 M NaOH. A 2.0 ml aliquot of the heptane phase was transferred to a scintillation vial and 10 ml of scintillation fluid (770 ml paradioxane, 770 ml xylene, 460 ml absolute ethanol, 10 g 2,5-diphenyloxazole, 100 mg α-naphthylphenyloxazole, 160 g napthalene) was added. Counting efficiency was approximately 65% as determined by internal standardization with (^{14}C) benzoic acid. Blank determinations always yielded less than 2% of values with enzyme present.

Glyceride synthesizing activity is expressed as micromoles of fatty acid esterified per gram tissue or per milligram of extractable protein. Kinetic data were derived from Lineweaver and Burk plots (14). Protein was determined by the method of Lowry et al. (15).

In some instances the products were extracted with chloroform-methanol (2:1 v/v) and the extracts applied to a silica gel chromatogram sheet (Eastman Kodak No. 6061, Rochester, N.Y.). Neutral lipids were separated by developing the chromatogram with hexane-ethyl ether-acetic acid (80:20:1 v/v) and identified according to Randerath (16). Polar lipids were separated by developing with chloroform-methanol-ammonium hydroxide (75:25:4 v/v). Two-way development separated incorporated palmitate from its salts. A neutral lipid standard (Sigma Chemical Co., St. Louis, Mo.) was co-chromatographed with all neutral lipid separations. Phospholipid identification was further facilitated by the use of the spray reagents molybdenum blue, Munier-Macheboeuf and ninhydrin (16). Lipid classes were located on

TABLE II

Subcellular Localization of Bovine Mammary Glyceride Synthetase Activity[a]

Fraction	Total activity	Protein[c]	Specific activity[d]
Part I			
800 x *g* Supernatant	238.0	14.2	16.8
80,000 x *g* Supernatant	2.0	9.8	0.2
80,000 x *g* Pellet	231.0	5.1	45.3
Resuspended 80,000 x *g* Pellet:			
12,000 x *g* Supernatant	52.0	1.8	28.0
12,000 x *g* Pellet	198.5	2.7	73.5
Part II			
100,000 x *g* Supernatant	21.1	4.6	4.6
100,000 x *g* Pellet	248.6	2.2	113.0
Recombination of 100,000 x *g* supernatant and pellet	367.4	6.8	54.0

[a]The values shown are averages of duplicate incubations. Parts I and II were conducted with tissue from different animals. Conditions of assay were those shown in Table I, except that enzyme source was varied as indicated.

[b]10^{-9} Moles palmitate esterified per hour per milliliter of fraction assayed.

[c]Milligram of extractable protein per milliliter of fraction assayed.

[d]10^{-9} Moles palmitate esterified per hour per milligram of protein.

the chromatogram sheet, cut out, and scraped into a vial for liquid scintillation counting. Areas of equal size from other portions of the plate were also scraped and counted in a liquid scintillation counter with 10 ml of scintillation fluid (10.5 g 2,5-diphenyloxazole, 450 mg *p*-bis-2(4-methyl-5-phenyloxazolyl-benzene), and 150 g naphthalene, 1500 ml paradioxane, 300 ml water).

RESULTS AND DISCUSSION

Cofactor and pH Requirements

Glyceride formation was highly dependent upon ATP, coenzyme A (Fig. 1 and 2) and α-glycerolphosphate and was stimulated by $MgCl_2$ and NaF. Incorporation without adding the latter three was 30%, 47% and 80%, respectively, of that when added in amounts indicated in Table I. Alpha-monopalmitin was not an effective acyl acceptor indicating a lack of the monoglyceride pathway. Addition of NADH, glucose-6-phosphate, or glutathione did not increase palmitate esterification. Bovine serum albumin (BSA) and dithiothreitol (DTT) were stimulatory but the stimulations were not additive until ATP was increased from 3.5 to 10.5 mM (Fig. 1). With the exception of DTT, these cofactor requirements are similar to those reported for rat (2), goat (8), and guinea pig (4,5) mammary tissue. Increasing CoA concentrations above 0.4 mM had no effect upon palmitate esterification (Fig. 2). Dithiothreitol probably protected enzyme rather than CoA sulfhydryl groups since it did not alter the CoA requirement. Addition of 2.75 mM DTT during homogenization did not influence esterification provided DTT was present in the incubation mixture.

A pH optimum of 7.2 to 7.3 was observed which is similar to that for goat mammary tissue (8). Phosphate buffer provided a more favorable medium for glyceride synthesis than did Tris buffer. Either buffer maintained pH within 0.1 unit during a 60 min incubation.

Kinetics of Palmitate Esterification

No palmitate was esterified by homogenates that had been boiled 1 min. The esterification rate of palmitate followed a somewhat sigmoidal pattern between 0 and 12 mg tissue per milliliter of incubation mixture (Fig. 3). This pattern is typical of reactions employing a micellar substrate (palmitate). Inhibition caused by detergent properties of palmityl CoA depend upon the protein to detergent ratio in the incubation mixture (17). Esterification of palmitate increased linearly between 3 and 9 mg of tissue per milliliter of incubation mixture and was linear with time to 60 min (Fig. 4). The substrate saturation curve for palmitate followed a hyperbolic form (Fig. 5). Reciprocal enzyme velocity departed from linearity at high substrate concentrations, demonstrating non-correspondence due to substrate inhibition

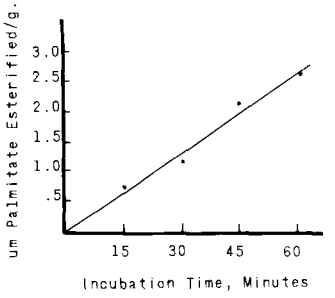

FIG. 4. Palmitate esterification as a function of length of incubation period. Conditions of assay were those shown in Table I, except that incubation time was varied as indicated.

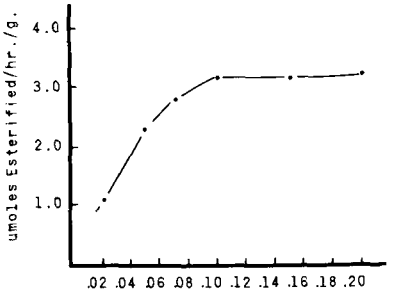

FIG. 5. Palmitate esterification in response to increasing concentration of palmitate in the incubation mixture. Each value represents the average of three determinations on the same homogenate. Conditions of assay were as shown in Table I except that palmitate was varied as indicated.

(18). An apparent Km of 0.13 mM determined in these studies was similar to a 0.17 mM palmitate Km reported for fatty acid esterification in rat adipose tissue (19). A maximum velocity (Vmax) of 7.89 μmoles palmitate esterified/hr/g tissue was obtained.

Subcellular Localization of Glyceride Synthetase Activity

Glyceride synthesis was associated with the particulate fraction of homogenized bovine mammary tissue (Table II), in agreement with previous findings for goat and guinea pig mammary tissue (4,5,8). The 12,000 x *g* pellet contained most of the particulate activity in agreement with reports on goat mammary gland (8) and rat adipose tissue (20). Guinea pig glyceride synthetase activity was divided equally between mitochondria and microsomes (4) whereas this activity in cat intestinal mucosa was predominantly microsomal in origin (21).

Glyceride synthesis by particles from rat liver was stimulated by addition of the 100,000 x *g* supernatant probably due to its soluble phosphatidate phosphohydrolase (EC 3.1.3.4) (22,23). A particle-free fraction from mammary tissue stimulated glyceride synthesis 36.2% (Table II). This stimulation was not as great as that reported for liver (22), but similar to that reported for intestinal mucosa (24). Since the monoglyceride pathway is not stimulated by addition of the particle free supernatant (22), the stimulation of esterification in this system provides evidence for the operation of the glycerol-3-phosphate pathway of glyceride synthesis. The lack of a larger stimulation of palmitate esterification by the 100,000 x *g* supernatant may be due to the presence of a particulate bound phosphatidate phosphohydrolase (23,24). Fluoride has inhibited phosphatidate phosphohydrolase and reduced palmitate esterification (22), but omission of NaF from the incubation mixture did not increase palmitate esterification in these studies.

Characterization of Product

The distribution of (^{14}C) palmitate in neutral and polar lipids following incubation was investigated (Table III). Most of the ^{14}C in neutral lipids was found in mono-, di- and triglycerides. A portion of the ^{14}C that migrated between the monoglycerides and diglycerides could not be identified by comparing with neutral lipid standards. This may have been a diglyceride consisting of an endogenous short chain fatty acid and a (^{14}C) palmitate. The 1,3-diglyceride, which may have resulted from isomerization of the 1,2-diglyceride (11),

TABLE III

Distribution of (^{14}C) Palmitate in Mammary Lipid Classes[a]

Lipid class	CPM[b]	Per cent of total esterified ^{14}C palmitate
Phospholipids	152	14.0
Monoglycerides	230	21.0
Unidentified	158	14.5
1,2-Diglycerides	230	21.0
1,3-Diglycerides	106	9.7
FFA	6646	—
Triglycerides	216	19.8
Cholesterol esters	0	0

[a]Cofactors were as in Table I except for ATP (7.0 mM) and DTT (O). Incubations were for 60 min at 37 C, pH 7.2, 0.4 ml of 800 x *g* supernatant from a 1:8 mammary homogenate. Similar results were obtained in three trials with the same tissue. Reaction was terminated by extracting the incubation mixture with chloroform-methanol (2:1 v/v). Lipid classes were separated by thin layer chromatography.

[b]Includes all CPM found between origin and solvent front of the thin layer sheet.

TABLE IV

Palmitate Esterification into Di- and Triglycerides as a Function of Time[a]

	Incubation time, min					
Glyceride class	15	30	45	60	120	150
Diglycerides, %	57	54	51	42	42	42
Triglycerides, %	43	46	49	58	58	58
Total nmoles Palmitate esterified	1.5	3.0	5.2	9.4	20.4	27.5

[a]All determinations were conducted on the same tissue homogenate. Cofactors and concentrations were as given in Table III. Enzyme source was 0.2 ml of a 1:8 mammary homogenate. Incubations were conducted for the time indicated at 37 C. Reactions were terminated at the appropriate time and lipids were heptane extracted and separated by thin layer chromatography as described in Methods and Materials.

produced a larger and more intense spot on the chromatogram than did the 1,2 form but contained half as much (^{14}C) palmitate (Table III). Lipolysis of diglycerides could account for the relatively high activity of the monoglycerides although the F^- in the incubation should have prevented extensive lipolysis (25). Bovine mammary cell cultures also incorporated large quantities of glycerol into monoglycerides (11).

Fourteen per cent of the esterified (^{14}C) palmitate was detected in phospholipids by this extraction procedure and 86% in neutral lipids. Phospholipid formation by this system was somewhat less than that with guinea pig mammary tissue (5), rat liver mitochondria (26) and rat adipose tissue (20). Yet, its composition was similar to that in cow mammary tissue (27). The major classes of phospholipids incorporating (^{14}C) palmitate in this system were phosphatidyl ethanolamine (39% of total polar lipid) and phosphatidyl choline (16%) and sphingomyelin (14%). Phosphatidic acid was detected using a standard but similar to other reports of lipid synthesis by bovine mammary tissue the amount was very small and difficult to detect (9,11). Phosphatidate phosphohydrolase may be extremely active in mammary tissue, preventing an accumulation of phosphatidic acid.

Distribution changed with incubation time. Diglycerides contained the greatest amount of (^{14}C) palmitate from 15 to 45 min (Table IV), but after 45 min, triglycerides contained 58% of the esterified (^{14}C) palmitate. This value is greater than that reported for goat, 24% (8), and about the same as that for guinea pig or rat, 57-63% (2,4), mammary tissue. Although the total incorporation of palmitate proceeded linearly to 150 min, the ratio of palmitate esterified into di- and triglycerides remained constant after 60 min. The possibility that excess acyl acceptor (glycerol-3-phosphate) masked the true extent of triglyceride formation was tested by incubating for 30, 60, 90 and 120 min with acyl acceptor limited (5.0 mM) or omitted. With palmitate in excess, triglyceride formation should be favored. However, decreasing acyl acceptor decreased total palmitate esterification and the relative per cent palmitate esterified into triglycerides was not increased.

The third acylation may have limited triglyceride formation by this system. In the phosphatidic acid pathway, the phosphate on carbon-3 must be removed by phosphatidate phosphohydrolase prior to the third acylation (23). Phosphatidate phosphohydrolase can be inhibited by NaF (28), but its absence did not increase the relative extent of triglyceride formation by this system above that noted in Table IV. Yet, total palmitate esterification was increased by NaF additions.

Another explanation for the paucity of triglyceride formation is suggested from the observation that short chain fatty acids (C_4 to C_{10}) occupy approximately 90% of the 3 position of ruminant milk fat triglycerides (29-31). Diglycerides of ruminant milk fat and mammary tissue are low in short chain fatty acid content, in comparison to triglycerides (27). Butyrate comprises approximately 10 mole per cent of the fatty acids in milk fat triglycerides and may be acylated to a 1,2-diglyceride acceptor forming triglycerides containing only one butyric residue per mole (27,32,33). Thus, the esterification of a short chain fatty acid, in particular butyrate, to a long chain diglyceride may complete the synthesis of a portion of milk fat. (^{14}C) Butyrate was not esterified significantly (0.04 μmoles/hr/g compared to 3.0 μmoles/hr/g for palmitate) by this in vitro system. The same system that esterified palmitate, stearate, oleate, linoleate and linoleneate esterified butyrate at only 1% to 2% of the rates observed for the long chain fatty acids (34). Butyrate

was not appreciably esterified by crude homogenates, tissue slices or fresh milk. The β-hydroxy form of butyrate was no more readily utilized than butyrate. None of the cofactors inhibited butyrate esterification. Carnitine, guanosine triphosphate, α-monopalmitin, NADPH, and a lipid extract of mammary tissue containing a variety of endogenous acyl acceptors did not stimulate butyrate incorporation. Numerous attempts to arrive at in vitro conditions conducive to butyrate esterification were unsuccessful (34). Pynadath and Kumar (8) and Woods (35) have reported similar negative results on butyryl CoA and butyrate esterification by goat mammary tissue. Their results cannot be explained by oxidation of butyrate, failure of butyrate to be activated to its CoA derivative (8) or the lack of a specific 1,2-diglyceride acceptor (35).

Although experimental evidence is lacking, short chain fatty acid esterification may be intimately related to fatty acid synthesis (36). Acyl carrier protein, known for its role in fatty acid synthesis (37), also functions in bacterial fatty acid esterification of glycerol-3-phosphate (38-41). Rao and Johnston (42) have demonstrated the formation of a protein-bound form of CoA from hamster intestinal mucosa that participated in fatty acyl transfer reactions. The exact nature of this compound has not been determined..

The inability of the in vitro system to form a product identical to milk fat suggests that portions of the fatty acid esterification mechanism by the mammary gland is governed by control mechanisms as yet undefined.

ACKNOWLEDGMENTS

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The Effect of Prolonged Fasting and of Glucose Refeeding on Rat Serum and Liver Lipid Levels

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ABSTRACT

The effects upon serum lipids of prolonged fasting of rats followed by refeeding with glucose or chow was studied. Fasting caused a decrease in the levels of serum triglyceride, phospholipid and cholesterol ester, while the level of total cholesterol remained unchanged. Refeeding 20% glucose in water for one day after three days of fasting caused a significantly greater decrease in serum lipid levels than did an additional day of fasting, while refeeding chow for one day restored the serum lipid levels to normal. Seven days of fasting or one day of glucose refeeding following three days of starvation resulted in almost complete disappearance of all serum lipoprotein bands on paper electrophoresis. After four days of refeeding glucose to rats previously fasted for three days the serum lipid levels and lipoprotein patterns approached those seen in fed animals. Serum free fatty acids increased upon fasting, but after seven days of starvation their concentration dropped to that observed in the fed rats. Refeeding for one day with glucose or chow reduced the serum free fatty acid concentration to less than half of the starvation levels. Four days of starvation caused a great decrease in liver weight without causing very pronounced changes in the content of phospholipid and triglyceride in the whole liver; the cholesterol content, notably esterified cholesterol, decreased. Glucose feeding for one day after three days of starvation, while increasing liver weight, did not cause any appreciable change in the hepatic lipid content. Following the three days of starvation lipogenesis from glucose by liver slices is restored to the same extent by refeeding glucose or chow for one day. The role of liver, adipose tissue and diet in supplying fatty acids for serum lipids following fasting is discussed.

INTRODUCTION

In the postabsorptive state the liver is the principle source of plasma triglyceride and phospholipids which are secreted as lipoproteins. These lipids have been stored in the liver or are newly synthesized using fatty acids obtained by lipogenesis from nonlipid sources (1) or from serum free fatty acids originating in adipose tissue. As the period of fasting is prolonged, lipogenesis is inhibited (2) so that adipose tissue indirectly becomes the main source of serum esterified fatty acids. Most cholesterol esters are formed in the plasma by the action of lecithin cholesterol acyltransferase which utilizes fatty acids from the plasma lecithin (3) and thus are also indirectly dependent on the fatty acid supply. It is known that feeding a large glucose load to fasted animals inhibits the flux of free fatty acids from adipose tissue into plasma (4,5), while the acute administration of carbohydrate to rats which have been fasted overnight or for 24 hr decreases the serum triglyceride levels (2,6,7). These observations have led Baker et al. (2) to stress the importance of free fatty acid mobilization for the maintainance of serum triglyceride levels after relatively brief fasting period followed by acute refeeding. After three days of fasting the reserve of fatty acid in adipose tissue appears to be diminished, although not depleted, since Bragdon et al. (6) found a decrease in the serum triglyceride, but not in the phospholipid level at this time.

We have examined the dependence of plasma lipid levels upon free fatty acid mobilization by two approaches. First, we have determined the extent to which the various plasma lipids would decrease when fat reserves are further diminished by a prolonged period of fasting of seven days. Second, we have studied whether hepatic lipogenesis, stimulated by glucose refeeding for several days following three days of fasting, would be adequate to maintain or restore plasma lipid levels. If lipogenesis is not increased sufficiently by glucose, one might anticipate that refeeding glucose to fasted rats could cause a fall in plasma lipids, paralleling that noted by Baker et al. (2) in acutely refed rats. This hypothesis was tested to provide some insight into the effects of prolonged fasting on serum lipids and on the contributions of the diet, adipose tissue and liver, respectively, to the serum lipid concentrations and lipoprotein pattern.

MATERIALS AND METHODS

Male hooded rats weighing 250-300 g were used throughout these experiments. The rats were maintained on a diet of Purina chow prior to the onset of fasting. Blood samples were taken from the tip of the tail under light ether anaesthesia. Serum triglycerides were determined by the method of Van Handel and Zilversmit (8) and free and total cholesterol by the method of Sperry and Webb (9). Lipid phosphorus was analyzed by the procedure of Fiske and SubbaRow (10). Electrophoresis of serum lipoproteins was performed in a Durrum-type paper electrophoresis cell for 16 hr at 8 mamps using 1% bovine albumin-barbital buffer pH 8.6 (ionic strength: 0.075). The paper strips were dried at 100 C and stained in a 60% ethanolic solution saturated with oil red O. Hematocrit was determined in an International microcapillary centrifuge. Serum free fatty acids were determined by the method of Dole and Meinertz (11).

To determine hepatic lipids the liver was removed and flushed with cold 0.9% NaCl, homogenized in 0.25 M sucrose and the lipids extracted by the method of Folch et al. (12). The lower phase was evaporated under nitrogen. The residue was redissolved in chloroform and aliquots taken for triglyceride (8) and lipid phosphorus (10) determinations. The remainder of the chloroform extract was dried and the cholesterol extracted from the residue with isopropanol. Free and total cholesterol were then determined by the method of Sperry and Webb (9).

Liver slices were prepared with a Stadie-Riggs microtome. Slices containing a total of 400-500 mg tissue were incubated in Krebs-Ringer bicarbonate buffer, pH 7.4 containing 4% bovine serum albumin, Fraction V (Nutritional Biochemical Corp., Cleveland, Ohio), 4 mM U-^{14}C-glucose containing 1.5 x 10^6 cpm in a total volume of 4 ml per flask. The gas phase was 95% O_2, 5% CO_2. After 1 hr of incubation at 37 C the contents of the flask were extracted by the procedure of Folch et al. (12), dried, redissolved in petroleum ether and an aliquot assayed by liquid scintillation counting. The remainder was saponified in 0.5 N alcoholic KOH and the nonsaponifiable lipid extracted. The alcoholic KOH was then acidified and the lipids extracted with heptane and counted.

RESULTS

In order to study the effects of prolonged fasting and refeeding with glucose or chow upon the level of serum lipids, three series of experiments were carried out, each using six

TABLE I

Influence of Fasting on Serum Lipid Levels[a]

Days of starvation	Animal weight, g	Hematocrit, %	Lipids, mg/100 ml serum					
			Triglyceride	Phospholipid	Total	Cholesterol		Ratio, free/ esterified
						Free	Esterified	
0	271 ± 6	40.8 ± 1.5	82.8 ± 6.6	171 ± 11	56.1 ± 5.8	16.2 ± 0.7	39.9 ± 4.4	0.39 ± 0.06
1	247 ± 6	41.4 ± 1.1	58.6 ± 3.8	160 ± 9	57.6 ± 3.2	19.0 ± 1.2	38.6 ± 3.1	0.46 ± 0.05
3	225 ± 9	39.3 ± 1.2	43.0 ± 3.6	124 ± 4	58.4 ± 3.3	20.9 ± 1.3	37.5 ± 3.4	0.56 ± 0.06
4	213 ± 6	37.8 ± 1.5	38.9 ± 4.8	118 ± 5	56.4 ± 3.1	21.3 ± 1.2	35.1 ± 3.2	0.60 ± 0.07
7	189 ± 4	36.7 ± 1.9	20.3 ± 2.3	106 ± 5	54.2 ± 3.8	24.3 ± 2.5	29.9 ± 2.8	0.82 ± 0.11

[a]Values are means from six animals ± S.E.M.

TABLE II

Influence of Starvation Followed by Glucose Refeeding on Serum Lipid Levels[a]

			Lipids, mg/100 ml serum					
						Cholesterol		
	Animal weight, g	Hematocrit, %	Triglyceride	Phospholipid	Total	Free	Esterified	Ratio, free/ esterified
Days of starvation								
0	277 ± 10	41.5 ± 1.5	71.9 ± 4.8	176 ± 9	54.1 ± 4.4	15.8 ± 2.0	38.3 ± 2.7	0.41 ± 0.04
1	263 ± 14	41.7 ± 1.5	46.6 ± 4.8	173 ± 8	55.3 ± 2.1	16.6 ± 1.8	38.7 ± 2.4	0.43 ± 0.07
3	236 ± 10	38.5 ± 1.9	34.8 ± 4.6	122 ± 5	52.5 ± 2.5	18.7 ± 2.1	33.8 ± 2.3	0.55 ± 0.04
Days of refeeding								
1	250 ± 9	33.5 ± 1.9	13.3 ± 2.3	46 ± 3	18.2 ± 1.6	8.9 ± 0.6	9.3 ± 1.1	0.84 ± 0.07
4	229 ± 12	34.3 ± 1.2	47.3 ± 5.2	117 ± 5	43.2 ± 3.8	17.8 ± 3.3	25.4 ± 1.5	0.63 ± 0.08

[a]Values are means from six animals ± S.E.M.

rats. In the first series the animals were fasted for seven days. The serum lipid levels and other relevant data are shown in Table I. During this period of starvation the animals showed a considerable loss of weight and a slight decrease in hematocrit. The serum triglyceride level continued to decrease throughout the seven day fasting period. The decrease in the serum phospholipid level became evident by the third day, but was not as marked as that seen in triglycerides. There was little change in the level of serum cholesterol, a finding also reported by Eaton and Kipnis (7) after two days of fasting. However, a gradual increase in free with a concomitant decrease in esterified cholesterol, resulting in a rise in the ratio of free to esterified cholesterol over the experimental period, was noted. Some of these results differ from those of Bragdon et al. (6) who reported an increase in total cholesterol but no change in phospholipid level in the sera of rats fasted for three days. The reason for these differences is not readily apparent.

In the second series of experiments six animals were fasted for three days, then allowed to drink, ad lib., water containing 20% glucose. The animal weights and levels of serum lipids during fasting and four days of the glucose refeeding are shown in Table II. It will be noted that when animals were on the glucose regimen their weight remained stable but hematocrit decreased. One day after refeeding with glucose there was a marked decrease in the serum level of triglyceride, phospholipid and cholesterol, especially esterified cholesterol. This decrease was significantly greater ($p<0.001$) than that seen in starvation (contrast with day 4, Table I). Thus, the decrease in serum triglyceride levels following glucose refeeding previously reported after overnight fasts (2,6-8), also applies following three days of fasting and can be extended to phospholipid and cholesterol levels. However, by the fourth day of the glucose regimen the serum lipid levels had risen, resembling those seen before glucose refeeding.

To provide additional comparison with prolonged starvation a third group of animals was fasted for three days, then restored to the chow diet. Analyses of the resulting serum lipids are shown in Table III. In contrast to glucose refeeding, within one day of the chow diet the serum triglyceride and phospholipid levels approached their normal (prefasting) level. Throughout the entire period there was little change in the total or the ratio of free to esterified cholesterol.

The effect of refeeding glucose or chow upon the serum free fatty acid levels was also investigated. Twelve animals were fasted for three days then divided into three groups of four animals each. One group continued to fast for an additional four days (Table IV, group 1) corresponding to the protocol used in Table I. Group 2 was allowed 20% glucose in their drinking water after the three days of fasting, corresponding to the regimen represented in Table II, while group 3 was allowed chow after the fasting period, corresponding to the conditions shown in Table III. The results, shown in Table IV, illustrate that there is a significant rise in the level of serum free fatty acids, reaching a maximum after three days of fasting then returning towards the normal levels after an additional four days of fasting (group 1). In group 2 one day of glucose refeeding decreased the free fatty acid level to less than 50% of the fasted level. Refeeding with chow (group 3) produced a dramatic drop in the free fatty acid level to one third of the prefasting level, returning to normal by the fourth day.

The alterations in serum lipids following the various dietary regimens are reflected by changes in the electrophoresis pattern of the serum lipoproteins, shown in Figure 1. After four days of starvation, the β, pre-β and most of the α-lipoprotein bands are still visible but have disappeared by the seventh day leaving only the pre-α-band. When the animals are refed glucose for one day following three days of fasting a disappearance of the pre-β and α-bands is noted. This correlates with the decrease in serum lipids reported in Table II and resembles lipoprotein pattern seen after seven days of fasting. By the fourth day of glucose refeeding the lipoprotein pattern was normal although the staining of the bands was lighter. One day of refeeding with chow restored the normal lipoprotein pattern.

The effect upon the hepatic lipids of three days of fasting, followed by refeeding for one day with glucose was also studied. The data are presented in Table V. Fasting produced a marked loss in liver weight which was only partially restored by refeeding glucose. After three days of fasting the hepatic levels of triglyceride rose somewhat, phospholipids fell slightly, and cholesterol decreased by almost 50%. These lipid levels were not significantly altered by refeeding glucose for one day. It will be noted that there is no apparent relationship between the changes in the hepatic and serum (Tables II and III) lipid levels.

Tepperman et al. (13) have shown that following a 48 hr fast, refeeding either sucrose or a balanced diet restored hepatic lipogenesis from glucose to almost the same extent. Since our protocol differed somewhat (three days fasting followed by refeeding glucose) an attempt was made to determine if our conditions can also

TABLE III

Influence of Starvation Followed by Purina Chow Refeeding on Serum Lipid Levels[a]

	Animal weight, g	Hematocrit, %	Lipids, mg/100 ml serum					
						Cholesterol		
			Triglyceride	Phospholipid	Total	Free	Esterified	Ratio, free/esterified
Days of starvation								
0	256 ± 15	42.0 ± 1.8	83.6 ± 7.7	183 ± 7	54.7 ± 3.8	16.7 ± 2.4	40.7 ± 3.4	0.41 ± 0.08
1	244 ± 16	42.5 ± 1.9	52.4 ± 4.6	173 ± 6	57.0 ± 4.0	16.7 ± 3.4	40.3 ± 2.5	0.41 ± 0.04
3	226 ± 10	38.2 ± 1.5	38.8 ± 5.0	136 ± 9	54.1 ± 2.6	18.8 ± 1.8	35.3 ± 1.5	0.47 ± 0.02
Days of refeeding								
1	240 ± 10	41.0 ± 1.4	62.7 ± 8.1	145 ± 8	56.4 ± 4.5	17.2 ± 2.0	39.2 ± 5.2	0.45 ± 0.04
4	258 ± 10	41.0 ± 2.4	75.3 ± 7.6	180 ± 8	57.0 ± 6.3	17.8 ± 2.4	39.2 ± 5.7	0.42 ± 0.04

[a]Values are means from six animals ± S.E.M.

TABLE IV

Influence of Fasting and Refeeding on Serum Free Fatty Acid Levels[a]

Days	Free fatty acid, μ Eq/liter serum		
	Group 1, fasted	Group 2, fasted, refed glucose	Group 3, fasted, refed Purina chow
0	440 ± 25	418 ± 31	419 ± 32
1	681 ± 50	714 ± 52	728 ± 56
3	808 ± 67	792 ± 77	751 ± 70
4	808 ± 87	309 ± 52	144 ± 27
7	524 ± 21	466 ± 23	454 ± 34

[a]Groups 1, 2 and 3 followed fasting and refeeding regimers described in Tables I, II and III, respectively. All values in each group are means from four animals ± S.E.M.

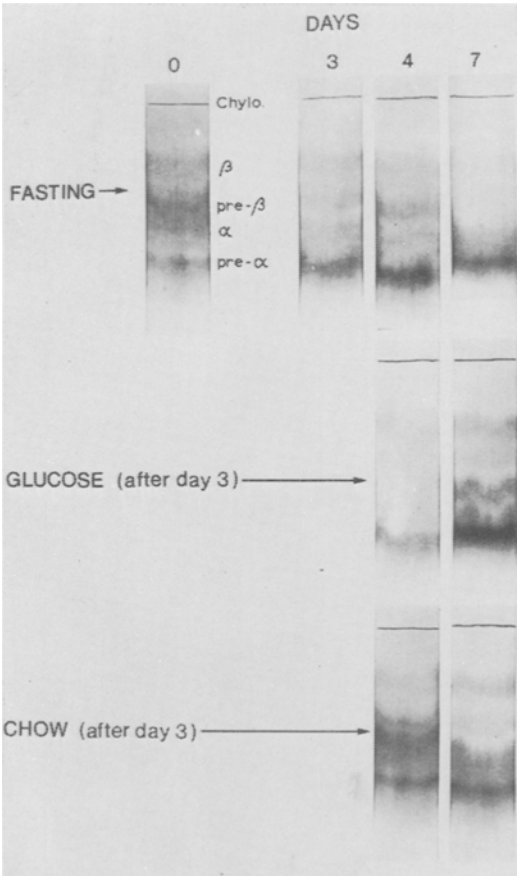

FIG. 1. Electrophoretic patterns of serum lipoproteins from fasted and glucose or chow refed rats.

cause a restoration of lipogenesis in the liver. Lipogenesis from glucose-U-^{14}C by tissue slices prepared from livers of animals which had been fasted for four days (group 1) or refed either glucose (group 2) or chow (group 3) for 24 hr was estimated. The liver slices were incubated with bovine serum albumin to avoid inhibition of lipogenesis by free fatty acids which might accumulate in the tissue, since esterification is inhibited in livers of fasted animals (14). The data are shown in Table VI. There was no detectable glycogen in the livers of the fasted animals but refeeding with glucose or chow elevated the level of glycogen to the same degree. Lipogenesis from fasted animals was negligible, the incorporation of glucose being limited to the glycerol moiety of the lipid, presumably for the esterification of the free fatty acids present in the tissue or associated with the bovine serum albumin. On the other hand, active lipogenesis from glucose was noted in livers of animals refed glucose or chow, the former being slightly greater ($p<0.05$). Esterification, as indicated by incorporation into the glycerol moiety, was approximately equal in the livers from animals refed the two types of dietary regimens. The ability of a diet limited to glucose to initiate lipogenesis is also indicated by the observations that after four days of glucose refeeding the serum lipoprotein pattern and lipid levels have returned towards normal (Table II and Fig. 1).

DISCUSSION

During the prolonged (7 day) fast there is a gradual decrease in the serum triglyceride, phospholipid and cholesterol ester levels, while the free fatty acid level, which had been elevated at first, returns to normal. This phenomenon can be explained by the hypothesis that as fasting progresses the supply of free fatty acid from adipose tissue is diminished so that the liver cannot maintain the level of esterified serum lipids. This decrease is reflected by a disappearance of the β, pre-β and most of the α-lipoprotein of the serum. When the 72 hr-fasted rats are refed with glucose for 24 hr a picture similar to that seen after the prolonged fast emerges. It would appear that hepatic lipogenesis, recommencing after glucose refeeding, is inadequate to compensate for the decrease in fatty acid mobilization. The fall in serum cholesterol levels, noted after glucose refeeding, can also be explained by the inhibition of fatty acid mobilization. Angel and Farkas (15) have pointed out the importance of adipose tissue as a storage depot of cholesterol which is mobilized, although somewhat more slowly, during free fatty acid release. Thus the serum cholesterol levels could be maintained during the prolonged fast but not after glucose refeeding.

TABLE V

Influence of Fasting and Refeeding on Liver Lipids[a]

Treatment	Liver weight, g	Lipids, mg/whole liver				
		Triglyceride	Phospholipid	Total	Cholesterol		Ratio free/esterified
					Free	Esterified	
Fed	11.3 ± 0.8	60.7 ± 6.3	242 ± 10	22.0 ± 0.7	17.1 ± 1.1	4.9 ± 0.6	3.5 ± 0.3
Fasted three days	6.3 ± 0.3	83.8 ± 3.3	200 ± 9	13.8 ± 0.6	11.4 ± 0.5	2.4 ± 0.2	4.5 ± 0.3
Fasted four days	5.7 ± 0.3	76.3 ± 5.4	182 ± 12	11.8 ± 0.5	9.6 ± 0.5	2.2 ± 0.3	4.7 ± 0.2
Fasted three days, then refed glucose for one day	8.1 ± 0.3	89.4 ± 4.0	200 ± 6	13.6 ± 0.3	10.7 ± 0.4	2.9 ± 0.2	3.8 ± 0.3

[a] All values are means from four animals ± S.E.M.

Refeeding a balanced diet (chow) for 24 hr restores the normal pattern of lipoprotein and levels of serum lipids in spite of a marked diminution in serum free fatty acid levels. Since hepatic lipogenesis does not appear to be greater following refeeding with chow than with glucose, it thus seems likely that the rapid restoration of the serum lipid levels and lipoprotein pattern due to the balanced diet may be due primarily to the intake of dietary lipid, although lipogenesis from amino acids may also play a role.

The foregoing analysis of our results is based on the assumption that the effect of glucose refeeding is due to a decrease in mobilization of all fatty acids, including linoleate, which, in mice on a fat free diet, disappears more readily from the liver than from adipose tissue (16). The resulting hepatic deficiency in linoleate may induce a decrease in the release of triglycerides. This has been demonstrated in perfused livers from rats chronically deficient in linoleate (17). On the other hand after four days of fasting, where fatty acid mobilization is not inhibited, the decrease in serum triglycerides would be less marked, as has been observed. Following four days of glucose refeeding the serum lipid levels return towards normal in spite of the absence of dietary essential fatty acids. While $\Delta 5,7,11$-eicosatrienoic acid is synthesized in linoleate deficiency and may meet some of the requirements for unsaturated fatty acids (18), it is doubtful if this can occur within four days, if the livers of mice on fat free diets can be used as a model (16).

Several additional qualifications to the foregoing analysis of our results must be borne in mind. It is possible that the failure of the serum lipid to return to normal on refeeding of glucose for one day may be due to an inability of the liver to synthesize the protein moiety of the lipoproteins. However, after a short period of fasting (16 hr), refeeding with glucose increased the ability of the liver to incorporate radioactive amino acids into the lipoproteins (19). The possibility that a shortage of essential amino acids may prevent synthesis of the protein moiety is unlikely, since a normal lipoprotein pattern is found after refeeding with glucose for four days. In addition, a defect in the secretion of lipoproteins due to decreased synthesis of the protein moiety might be expected to produce an accumulation of hepatic lipid, especially triglyceride. This is seen in various hepatotoxic conditions, e.g., CCl_4, ethionine, puromycin or orotic acid intoxication, where protein synthesis is inhibited. However, there is no change in the hepatic triglyceride level following the glucose refeeding, sug-

TABLE VI

Lipogenesis by Rat Liver Slices Following Fasting and Refeeding From Glucose-U-^{14}C[a]

Treatment	Incorporation into esterified lipids, cpm/100 mg tissue			Hepatic glycogen, mg/100 mg tissue
	Glyceride-glycerol	Glyceride fatty acids	Nonsaponifiable lipids	
Four days fasted	400 ± 10	10 ± 1	---	---
Three days fasted, then refed for one day with glucose	730 ± 80	150 ± 15	30 ± 3	3.4 ± .4
Three days fasted, then refed for one day with Purina chow	620 ± 60	110 ± 10	25 ± 3	2.8 ± .4

[a]Each value is the average of four animals ± S.E.M.

gesting that the balance between hepatic triglyceride accumulation and secretion as lipoprotein is maintained. Thus when the fatty acid load is smaller less lipoprotein is secreted. A second consideration in interpreting our results is the possibility that the decrease in serum lipids and lipoprotein levels following glucose refeeding might be due to an increase in the peripheral uptake of the triglycerides. Refeeding after short or long term fasts results in an increase in adipose tissue (20,21), but not plasma (7), lipoprotein lipase activity. Nikkila and Ojala (22) have shown that refeeding glucose after 18 hr of fasting slightly increases the outflow of endogenous plasma triglycerides, although generally the efflux was proportional to the plasma triglyceride concentration rather than the dietary state after the short term fasting. However, an increase in triglyceride uptake following glucose refeeding could not readily account for the lower phospholipid and cholesterol levels. Furthermore, while increased triglyceride uptake would account for the decrease or disappearance of the very low density lipoproteins (VLDL) it could not explain the decrease in the low density (LDL) and high density lipoproteins (HDL) of serum. Indeed, increased removal of triglycerides from VLDL results in an increase in HDL and LDL (23).

It is well established that during fasting fatty acids are supplied to the liver by mobilization from adipose tissue. Therefore, we concluded that it is likely that the fall in plasma lipids, which we have observed during prolonged starvation, resulted from a diminished capacity of adipose tissue to supply free fatty acids to the liver as some of the fat depots became depleted. Although hepatic lipogenesis from glucose was increased 15-fold following glucose-refeeding, hepatic lipogenesis from glucose was obviously not fast enough to restore the plasma lipids to normal during a one day refeeding period. Since lipogenesis was not further increased by refeeding a balanced diet, while the serum free fatty acid level was markedly diminished, it appears that the diet supplies the fatty acids and possibly the ketogenic amino acids to replenish the serum lipid levels and restore the lipoprotein patterns.

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Trans-6-hexadecenoic Acid and the Corresponding Alcohol in Lipids of the Sea Anemone *Metridium dianthus*

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ABSTRACT

Trans-6-hexadecenoic acid was found in polar lipids, triglycerides, wax esters and diacylglyceryl ethers of the sea anemone *Metridium dianthus* from Passamaquoddy Bay. The corresponding alcohol also apparently occurs in the wax esters of this species. The long-chain (C_{20}, C_{22}) monoethylenic alcohols reported for other species of sea anemones from neighboring waters were absent and the major alcohol and glyceryl ether chain both had 16:0 structures. The isomers of C_{18} and C_{20} monoethylenic fatty acids in polar lipids and triglycerides were unusual in their high proportion of the ω_7 isomer. These two lipids also contained higher proportion of the polyunsaturated fatty acids than the others.

INTRODUCTION

In the marine environment *trans*-6-hexadecenoic acid has been found in the fats of three marine turtles (1) and in lipids of the ocean sunfish *Mola mola* (unpublished results from this laboratory). The dietary factors common to these animals suggested an association with jellyfish (2), which are, unfortunately, very low in lipid (3) and not readily available in Nova Scotian waters except in late summer. Another member of the phylum coelenterata, the sea anemone *Metridium dianthus*, could be obtained locally and other anemones from the same area were reported to be rich (up to 30% dry weight) in lipid (4). Our investigation of this species has confirmed the presence of *trans*-6-hexadecenoic acid and also of the structurally related alcohol, *trans*-6-hexadecenol.

EXPERIMENTAL PROCEDURES

M. dianthus were obtained from the St. Croix estuary, Passamaquoddy Bay area of the Bay of Fundy through Maritime Biological Laboratories, St. Stephen, New Brunswick, in September and again in October, 1968. They were held in tanks with filtered seawater prior to sacrifice at various times from September to the following February.

Sacrifice of whole animals was effected by immersion for a few minutes in the methanol subsequently used for lipid extraction by the Bligh and Dyer method (5). Polar lipids were separated from neutral lipids on a polystyrene gel column (3). The recovered neutral lipids were separated by preparative thin layer chromatography (TLC) on silica gel into triglycerides, diacyl glyceryl ethers and wax esters (6). Triglycerides and polar lipids were transesterified by 30 min treatment with 5% BF_3 in MeOH (7). Water was added and esters recovered into petroleum ether which was given one water wash to remove mineral acids and MeOH.

The diacylglyceryl ethers and wax esters were saponified and the alcohol and glyceryl ether moieties were extracted into diethyl ether following procedure Ca-6b-53 of the Official and Tentative Method of the AOCS. The alcohols were acetylated with acetic anhydride and the glyceryl ethers were converted to chlorides with BCl_3 (8). The recovered fatty acids were converted to methyl esters with BF_3-MeOH.

Open tubular gas liquid chromatography (GLC), preparative GLC and TLC separations with plates coated $AgNO_3$-silica gel were essentially the methods used previously (1).

RESULTS

In several extractions, the lipid content of the *M. dianthus* studied varied from 0.3 to 1.3% (wet weight), the smaller specimens giving the higher values. The lipid was shown by TLC to contain in all cases mostly polar lipids with the same chromatographic characteristics as phospholipids, plus triglycerides, diacyl glyceryl ethers and free sterols. Gel column chromatography gave (by weight) recoveries of 50-60% as the polar lipid fraction, 15-20% as free sterols, and the remainder as the neutral fraction which contained roughly equal proportions of wax esters and triglycerides, with small proportions of diacyl glyceryl ethers.

The major object of this study, *t*16:1ω10, was identified in the methyl esters of fatty acids from all four lipid classes examined (Table I). Basic identification by comparative GLC behaviour on butanediol succinate (BDS) and Apiezon-L (AP-L) liquid phases was confirmed by isolation and IR examination (1). The complete GLC analyses of methyl esters of fatty

TABLE I

Composition in Weight Per Cent of Fatty Acids From Various Lipids of the Sea Anemone *Metridium dianthus*, and of Fatty Alcohols and Alkyl Glyceryl Ethers

Structure	Methyl esters of fatty acids from: Phospho-lipids	Triglycerides	Wax esters	Diacyl glyceryl ethers	Acetate esters of wax ester fatty alcohols	Dichloro derivative of alkyl glyceryl ether
14:0	.17	2.46	6.32	10.96	5.78	10.67
Iso 15:0	.04	.25	1.56	2.20	—	—
Anteiso 15:0	—	.17	.60	1.42	—	—
15:0	.09	.59	3.44	4.98	2.57	—
Iso 16:0	.06	.10	.46	.49	—	—
16:0	4.60	15.69	23.09	25.50	65.47	72.00
Iso 17:0	.26	.47	.45	.34	—	—
Anteiso 17:0	.13	.24	.23	.12	—	—
17:0	.29	1.24	.39	.56	3.22	—
Phytanic[a]					3.20	—
Iso 18:0	.09	.23	.60	.14	—	—
18:0	5.92	4.97	3.65	4.38	11.55	12.00
19:0	.17	.17	.46	.14	—	—
20:0	.10	.11	.30	.34	—	—
21:0	—	—	—	—	—	—
22:0	—	—	—	—	—	—
Total	11.92	26.69	41.55	51.57	91.79	94.67 Table I (2)
14:1ω9?	—	.25	1.58	5.84	—	—
14:1ω7	.37	.31	—	—	—	—
15:1ω8	.03	.59	1.25	1.56	—	—
t16:1ω10	.37	2.67	6.14	7.32	5.13	—
16:1ω7	.30	3.22	5.54	10.30	—	—
16:1ω5	.34	.95	.30	—	—	—
17:1ω8	.04	—	.60	1.93	—	—
18:1ω9	1.25	3.61	7.51	6.38	1.50	5.34
18:1ω7	1.02	3.41	1.37	1.17	1.58	
18:1ω5	.44	1.40	.30	.34	—	—
20:1ω11	—	.24	—	.14	—	—
20:1ω9	1.01	.92	.37	.20	—	—
20:1ω7	6.28	2.77	.55	.17	—	—
20:1ω5	.15	.45	.15	1.32	—	—
22:1ω13+11	.67	.86	—	.14	—	—
22:1ω9	3.16	1.27	—	.17	—	—
22:1ω7	.57	.24	—	.07	—	—
22:1ω5	.53	.11	—	—	—	—
Total	16.53	23.27	25.66	37.05	8.21	5.34
16:2ω6	.61	.11	2.12	.56	—	—
18:2ω6	.46	1.66	1.34	.34	—	—
20:2ω6	.89	1.02	1.12	—	—	—
22:2ω6	.43	—	—	—	—	—
Total	2.39	2.79	4.58	.90	—	—
16:3ω3	.22	.36	—	—	—	—
18:3ω3	.65	2.28	1.24	.45	—	—
20:3ω3	.30	.62	—	.07	—	—
Total	1.17	3.26	1.24	.52	—	—
18:4ω3	.28	5.45	.97	.14	—	—
20:4ω6	2.55	.44	1.12	.07	—	—
20:4ω3	.91	1.47	.76	.69	—	—
21:4ω2	.16	—	—	—	—	—
22:4ω6	4.24	.39	—	—	—	—
Total	8.18	7.75	2.85	.90	—	—
20:5ω3	27.14	14.31	17.18	2.75	—	—
22:5ω3	11.95	4.17	2.13	.43	—	—
Total	39.09	18.48	19.31	3.18	—	—
22:6ω3	19.01	17.59	4.35	4.88	—	—

[a] 3,7,11,15-Tetramethylhexadecane skeleton.

acids (Table I) include only fatty acids which could be plausibly identified with precision by comparison with commonly available standards, or with esters of fatty acids of marine oils from higher animals (9,10), or by hydrogenation of the whole ester mixture, or by isolation of saturates by $AgNO_3$-silica gel TLC. In each analysis of lipid fatty acid methyl esters a number of peaks which were completely unknown or could not be verified by at least two techniques were observed and these have been tabulated with equivalent chain length (ECL) values for the BDS open tubular columns operated at 170° and 40 psig helium (Table II). Some may by GLC breakdown products of alkenyl ether-derived dimethyl acetals of long chain aldehydes. These give multiple products on stainless steel open tubular columns (R.G. Ackman, unpublished observations). Two decimal places are given only to show minor components; accuracy is approximately within ±5% for major components and ±30% for minor components.

The glyceryl ether chlorides were compared on both BDS and AP-L columns with freshly prepared chlorides of selachyl (18:1), batyl (18:0) and chimyl (16:0) alcohols. In addition to the few recognizable major components (Table I) several substantial peaks were observed (Table II) which could not be identified due to lack of standards. The fatty alcohols from the wax esters, studied as acetates (Table I), could be more exhaustively compared with standards in the form of mixtures of alcohols prepared by reduction of marine oil fatty acids (11), but also included some unknown components (Table II). The component in the 16:1 region was unexpectedly found to correspond exclusively to a *trans*-6-isomer ($t16{:}1\omega10$) structure. As a proportion of total lipid (0.1-0.2%) the amount was too small to permit IR or oxidative fission studies, but on GLC its behaviour as the acetate was precisely analagous to that of the methyl esters of the $t16{:}1\omega10$ acid on BDS and AP-L, it formed only 16:0 alcohol on hydrogenation, and on $AgNO_3$-silica gel TLC it was in the correct position between the acetates of the saturated alcohols and the spots recovered and identified as $18{:}1\omega7$ and $18{:}1\omega9$.

The sterol recovered was found to consist of over 95% cholesterol by TLC and GLC analysis. Minor sterol components were not studied.

DISCUSSION

The presence of $t16{:}1\omega10$ in the fatty acids of all four lipid fractions suggest that it is part of a general fatty acid pool. The deposition in the fat of the marine turtles and ocean sunfish shows that, if available, it is ingested and included in depot and other fats by higher animals. As in the turtles, no corresponding $t18{:}1\omega10$ acid was observed in *M. dianthus*, so it is unlikely that $t16{:}1\omega10$ is metabolically active. Since the corresponding $t16{:}1\omega10$ alcohol is found in a high proportion relative to $18{:}1\omega9$ (or $\omega7$) in the alcohols, and to the exclusion of $16{:}1\omega7$, it is possible that the origin of this particular structure is in the alcohol form, with subsequent partial conversion to fatty acid as outlined in a recent review by Nevenzel (12). Oil drops dispersed in the tissue of coelenterata probably serve as depot fat (4), but we do not know if the wax esters observed in *M. dianthus* and other coelenterates (12,14) are included in this type of lipid. Possibly the $t16{:}1\omega10$ acid or alcohol structure has some function associated with the large surface area of these animals. Unfortunately, the diacyl glyceryl ether analysis on limited quantities of material did not permit identification of a corresponding ether-linked aliphatic chain which could originate in fatty alcohol (15), although the ECL value of a significant (8%) unknown component is approximately correct at 16.10 BDS. In ratfish (*Hydrolagus colliei*) liver oil, the C_{16} chain lengths, including $\omega10$ and $\omega8$ monoethylenic structures (presumably *cis*), were found in the alkyl diglyceride, but not in the alk-1-enyl diglyceride, as well as in the fatty acids of these two classes and of triglycerides (16).

A previous study (4) showed the lipids of the warm water anemone *Condylactis gigantea* to be dominated by 14:0 alcohol (71%) and 14:0 acid (51%), with a lesser proportion of 16:0 acid (31%). In the cold water anemones (Gulf of Maine) the lipids of *Bolocera tuediae* were quite different, although not different from *Actinostola callosa* also collected in the same area. Thus the 16:0 alcohol (10.5%) was of less importance than $20{:}1\omega9$ (27%) and $22{:}1\omega11$ (47%), while in the acids 14:0 and 16:1 (13% each) were less than $20{:}1\omega11$ (reported as 20%), $22{:}1\omega11 + 13$ (21%), and C_{24} materials reported as 22%. The present work with *M. dianthus* did not reveal any very long chain (C_{22}) fatty alcohols, although a C_{20} alcohol may have been present, and only modest proportions (<5% overall) of 20:1 and 22:1 acids. It is not apparent if these rather marked differences are associated with temperature effects (17) or are species differences.

The polar lipids of *M. dianthus* are presumably mostly phospholipids and might well follow the composition reported for *Anthopleura elegantissima* (18). The distribution of

TABLE II

Composition in Per Cent of Recorder Area of Components of Unconventional Structures From GLC Analyses of Mixtures of Methyl Esters of Fatty Acids, Acetates of Fatty Alcohols, and Dichloro Derivatives of Alkyl Glyceryl Ethers[a]

In methyl esters of fatty acids from								In acetate esters of wax ester fatty alcohols		In dichloro derivative of alkyl glyceryl ether	
Phospholipids		Triglycerides		Wax esters		Diacyl glyceryl ethers					
ECL	%	ECL	%	ECL	%	ECL	%	ECL	%	ECL	%
13.70	0.30	—	—	—	—	—	—	15.50	6.61	14.10	5.01
15.35	0.48	—	—	15.25	0.28	15.35	2.48	15.75	1.87	14.35	5.01
15.70	5.58	—	—	15.70	0.28	15.68	1.11	17.75	0.88	15.10	16.27
—	—	15.75	0.46	15.80	0.22	15.75	0.34	19.65	15.00	15.20	5.01
—	—	—	—	—	—	16.12	1.28	19.95	13.22	16.10	8.01
16.90	1.47	17.10	0.26	—	—	16.90	0.76	21.25	5.14	17.10	13.77
17.70	2.23	—	—	—	—	17.75	1.45	—	—	17.15	
17.80	0.32	—	—	—	—	—	—	—	—	—	—
17.90	0.72	17.95	0.40	—	—	—	—	—	—	—	—
18.10	0.68	18.10	0.40	—	—	—	—	—	—	—	—
18.30	0.56	18.40	0.26	18.40	0.28	—	—	—	—	—	—
20.70	0.35	20.65	0.93	20.70	13.66	20.68	30.98	—	—	—	—
24.15	1.50	—	—	—	—	23.40	0.53	—	—	—	—
24.20	0.41	—	—	—	—	—	—	—	—	—	—

[a]Equivalent chain lengths given for appropriate straight chain derivatives analyzed on BDS coated open tubular column at 170 C and 40 psig helium.

fatty acids in this fraction shows a resemblance to that of the polar lipids from the jellyfish *Cyanea capillata* (3). In comparison with the respective triglycerides, the polar lipids agree in overall low proportions of 14:0, 16:0, and 18:1 in low proportions of 22:6ω3 relative to 20:5ω3, and in higher proportions of 20:4ω6 and 22:4ω6. In both lipids the jellyfish showed higher proportions of longer chain normal saturated fatty acids such as 19:0, 20:0 and 22:0. Most of the details in the minor acids, and in the proportions of the isomers within the monoethylenic chain lengths, are very similar in the two respective types of lipids for *M. dianthus* and *C. capillata*.

The saturated and monounsaturated fatty acid components of the wax esters are in many respects quantitatively intermediate between those for the triglycerides and for the diacyl glyceryl ethers. The reason for this is not immediately apparent. Although the two 18:1 isomers, 18:1ω9 and 18:1ω7 are approximately equal in the phospholipids and triglycerides, there is a considerable excess of ω9 over ω7 in the fatty acid of the wax esters and diacyl glyceryl ethers. In one view this suggests de novo biosynthesis of 18:1ω9 for inclusion in these two lipids, whereas in the phospholipids and triglycerides the chain extension of 16:1ω7→18:1ω7 can be further extended to the unusual dominance of the 20:1ω7 among the 20:1 isomers. Alternatively, the proportion of 18:1ω9 to 18:1ω7 for the wax esters and diacyl glyceryl ethers is approximately that found in many marine lipids and these two acids could be of dietary origin. The accumulation of the 22:1ω9 isomer is unusual for marine lipids. Similar trends for C_{20} and C_{22} isomeric monoenoic acids in neutral alkoxy lipids and triglycerides of *H. colliei* liver oil seem to be affiliated with gradations among alk-1-enyl, alkyl and triglycerides (16). There was no significant amount of 16:1ω9 relative to 16:1ω7.

The wax ester fatty alcohols conform to the profiles observed in many other marine lipids (12). In detail, for example, weight per cent composition of a particular mullet (*Mugil cephalus*) roe sample (19) shows a striking similarity with 14:0 = 9.9%, 15:0 = 6.2%, 16:0 = 54.3%, 17:0 = 17% and / 18:0 = 6.4%. This extends to the 2:3 ratio of 18:1ω9 and 18:1ω7 reported in the same study for another mullet roe sample. It is interesting that the authors comment on wax esters as a phylogenetic vestige occurring in mullet only in early embryonic development.

Phytanic (3,7,11,15-tetramethylhexadecanoic) acid could not be detected in significant quantities, although possibly traces were present. Pristanic (2,6,10,14-tetramethylpentadecanoic) and 4,8,12-trimethyltridecanoic acids could not be detected.

The dihydrophytol was identified by GLC only, using an authentic reference material, and has no convenient explanation. It could be formed from phytol in herbivorous animals such as copepods and simply deposited with other alcohols on ingestion by the anemone. However in some molluscs the heavy intake of phytol is apparently disposed of by degradation to 4,8,12-trimethyltridecanoic acid (20). Possibly *M. dianthus* or another animal could dispose of ingested phytol by reduction to a saturated alcohol. If this is correct, it would be a nearly unique case of biohydrogenation, although *trans*-2-phytenic acid can be reduced by rat intestinal mucosa (21). In the jaw lipid of *Inia geoffrensis*, a freshwater dolphin, the wax esters contained no dihydrophytol (22).

The glyceryl ether composition suggests formation from the major fatty alcohol components, and in this resembles the *Inia* jaw lipid where diacyl glyceryl ethers were also a minor component relative to wax esters and triglycerides. In the analysis of *Inia* glyceryl ethers ditrimethylsilyl ether derivatives were used, and there was also strong evidence for odd-numbered alkyl chains such as 15:0, 17:0 and 17:1. These may, therefore, be present as suggested by the data of Table II based on the analysis of dichloro derivatives. Batyl alcohol may have been the dominant glyceryl ether in *B. tuediae* and *A. callosa* in association with the generally longer chain lengths in other lipids (4).

Cholesterol is frequently but not always the principal sterol in coelenterates (4,23,24).

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SHORT COMMUNICATIONS

Liver Triglyceride Synthesis Failure in Post-Spawning Salmon

ABSTRACT

The ability of liver of pink salmon, *Onchorhychus gorbuscha*, to synthesize triglycerides is lost after migration into fresh water and spawning. Triglyceride fatty acids are probably mobilized as cholesterol esters as energy sources in these starving fish. Total lipid decreases; cholesterol remains constant; and relative values of protein and water increase in the liver of the spawned out fish. Both hepatic lipogenesis and cholesterol synthesis decrease. Control values are given for river whitefish.

There appears a pronounced difference in liver triglyceride synthesis between ocean pre-spawning and river post-spawning pink salmon, *Onchorhynchus gorbuscha*. After entering fresh water and spawning these fish have essentially lost the ability to synthesize triglycerides in the liver. This correlates with observed liver lipid decrease and decreased rates of 1-^{14}C-acetate incorporation into lipid and cholesterol. Blood triglyceride decrease (from 375 to 71 mg/100 ml) has been reported (1) in post-spawning salmon of the same species, as evidence that these fish are starving. Low levels of liver microsomal azo-reductase activity and oxidation of ^{14}C-palmitic acid to $^{14}CO_2$ have been found as evidence for hepatic insufficiency (2). The characteristic demise of Pacific salmon after spawning has been recently summarized (3,4). What little information is available on lipid utilization in starving fish of other species has been recently reviewed (5). An elevated free fatty acid content of the blood has been noted in *Salmo gairdnerii* as well as increase of highly unsaturated fatty acids in their muscle. A decrease in phospholipid as proportion of total lipid was found in starved *Onchorhynchus tschawytscha* and *Gadus morhua*. However, studies involving ^{14}C-labeling of lipogenesis have been mostly restricted to mammals (6,7) and birds (8,9). Marked decreases in hepatic fatty acid and cholesterol synthesis have been repeatedly observed in individuals within these groups.

Pre-spawning ocean salmon were collected in Kisameet Bay, near Namu, British Columbia. They were obtained alive from commercial purse seiners. Post-spawning salmon were netted from the Atnarko river in Tweedsmuir county, near Bella Coola, British Columbia. Livers were excised from living fish, immediately thin sliced, weighed and placed in tissue culture flasks. Tissue medium "B" of

TABLE I

TLC[a] Results of Pre-Spawning Ocean and Post-Spawning River Pink Salmon[b]

		Pre-spawning ocean pink salmon, fresh weight excised liver slices, 550 mg		Post-spawning river pink salmon, fresh weight excised liver slices, 627 mg	
Spot No.	Compound	dpm	% Total dpm	dpm	% Total dpm
6	—	Trace	Trace	1500	29
5	Triglycerides	7500	36	28	1
4	—	1800	9	710	14
3	Cholesterol, etc.	3000	14	1400	27
2	—	1200	6	570	11
1	Phospholipids	7500	36	880	18
Total		21,000		5,110	

[a]Solvent system, petroleum ether-diethyl ether-glacial acetic acid (50:50:1).
[b]Extracted lipid, after 1-^{14}C-acetate-incorporation by excised liver, has been separated into components, then analyzed by scintillation counting (counting error of $\pm$ 2%).

TABLE II

Conversion of 1-^{14}C-Acetate to Lipid and Cholesterol in Liver, and Percentages of Liver Water, Lipid and Protein[a] and Cholesterol in the Salmon, *Onchorhychus gorbuscha,* and the River Whitefish, *Prosopium williamsoni*

Fish examined	μMoles of 1-^{14}C-acetate per gram protein) incorporated into		Total cholesterol as per cent of	As per cent in Liver		
	Lipid	Free cholesterol	Lipid	Lipid, dw%	Protein, dw%	Water
Ocean salmon	—	—	—	—	66(57-75)	70(69-71)
River pre-spawning salmon	100(14-190)[b]	1.9(0.2-3.9)	2.8(1.6-3.8)	9.9(2.1-20)	69(52-78)	76(72-81)
River post-spawning salmon	53(11-110)	0.5(0.0-1.7)	2.5(1.1-4.5)	3.2(2.5-4.3)	89(88-90)	82(80-83)
River whitefish	—	5.1(0.2-12)	2.9(1.8-4.6)	24(5.3-46)	77(71-90)	75(74-77)

[a]As dry weight (dw).
[b]All figures are averages from four to five separate fish. Ranges of values are given in parentheses.

D'Aoust (10) was used with addition of 4mM sodium acetate (as carrier for 3 x 10^{-5} mM 1-^{14}C-acetate; specific activity was 0.5-1.5 x 10^6 dpm/mmole acetate) and 30 mM glucose. Incubations were carried out for 4 hr (30 C, pH7.3) under a gas mixture of 90% O_2, 9%N_2, and 1% CO_2. The gas mixture was used to compare these results with those for cholesterol biosynthesis in deep sea fish (11). Lipid was extracted by the method of Bligh and Dyer (12). Many of the methods used here have been modified from Longmore et al. (13,14). Thin layer chromatography (TLC) (petroleum ether-diethyl ether-glacial acetic acid, 50:50:1) was used to identify lipid components. Part of the plate was sprayed with 40% H_2SO_4 and charred to locate spots which were then scraped and analyzed for ^{24}C-activity in a scintillation counter. 1-^{14}C-Acetate incorporation into lipid and cholesterol (precipitated as the digitonide) was also determined by scintillation counting. Percentage of total cholesterol was obtained by Liebermann-Burchard assay (15); protein content was estimated by the method of Lowry et al. (16). Technical difficulties precluded obtaining data on 1-^{14}C-acetate incorporation into liver and cholesterol of ocean salmon and lipid of river whitefish. All pre-spawning females, including the whitefish, were gravid. Dry weights were obtained by drying to constant weight at 50-60 C.

Results of TLC (Table I) illustrate the major differences in hepatic lipogenesis after migration into fresh water, fasting and spawning. Triglyceride synthesis essentially ceased (from 36% to 1% incorporation of 1-^{14}C-acetate. 1-^{14}C-acetate incorporation into liver dropped to one fourth the values for ocean salmon and phospholipid synthesis was reduced by one half. Increased incorporation into Spot 3 (cholesterol, etc.) shows presence of 1-^{14}C-acetate in other compounds there, since quantitative cholesterol precipitation showed decrease in cholesterol biosynthesis after fasting and spawning (Table II). Close to one third of the incorporation was in Spot 6 in the starved salmon. This spot includes nonpolar lipids such as cholesterol esters and hydrocarbons.

Total hepatic lipogenesis dropped to one half normal rates after spawning whereas cholesterol synthesis dropped to one fourth the pre-spawning level (Table II). Control river whitefish (*Prosopium williamsoni*) had an average of 10 times the post-spawning salmon cholesterogenic capacity. The salmon liver gained water, decreasd in lipid content and increased in relative amount of protein after spawning (Table II). Control river whitefish showed intermediate values. The salmon cholesterol to total lipid proportion was essentially constant despite starvation and spawning.

The remarkable loss of ability to manufacture triglycerides by the liver correlates with their overall decrease in the liver (TLC charring observation) and in the blood (1). It is clear that triglycerides are being utilized as a food reserve in these starving fish. This is reflected by decrease in liver lipid after spawning and the long period of fasting (Table II). Cholesterol is used to mobilize the fatty acids of triglycerides in the form of cholesterol esters. This has been shown in sardines (R.F. Lee and D. Puppione, in preparation). Thus, one would expect increased 1-^{14}C-acetate incorporation into cholesterol ester; the present TLC data (Table I) support this. The fact that the liver does not synthesize triglycerides explains the absence of low density lipoproteins in the serum of the fasted and spawned salmon (17). The site of the block in glyceride synthesis is probably at some

point between acetyl-CoA and fatty acids as in rats (18). Increased plasma free fatty acid levels have been observed (9) to precede decrease in hepatic fatty acid synthesis in the fasting chick. In *Salmo gairdnerii*, starving has also resulted in elevated blood free fatty acids (5). Free fatty acids may compete for coenzyme A, thereby limiting its availability for fatty acid synthesis. More work is needed to clarify these mechanisms in fish.

Despite reduction of cholesterol biosynthesis in response to spawning, the stable liver cholesterol values are evidence for participation in membrane structure, which may retain integrity despite other changes. Cholesterol values in these pre-spawning river pink salmon (70 mg/100 g tissue) are somewhat lower than reported values for river sockeye salmon (231 and 183 mg/100 g tissue) (19). Increase in liver water (Table II) correlates with observations of edema in tissues of migrating river salmon and measurements of positive interstitial fluid pressures (Alan Hargens, private communication). This also reflects relative decrease of other components, such as lipid and carbohydrates, which are used as energy sources. The increase of protein in the liver, especially in the post-spawning salmon, reflects proportional decrease in lipid and carbohydrate in response to energy demands.

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Cyanolipids of Kusum (*Schleichera trijuga*) Seed Oil

ABSTRACT

A cyanolipid, consisting of two fatty acid moieties esterified with 1-cyano-2-hydroxymethylprop-2-ene-1-ol, constitutes 58% of *Schleichera trijuga* (Kusum) seed oil.

Oil obtained from the seeds of *Schleichera trijuga* (Kusum) is an item of commerce in India. A member of the Sapindaceae, *S. trijuga* is a large tree native to that general section of Asia. Cyanogenetic materials were detected in this oil some time ago (1,2); however, the latest reports concerning these interesting constituents (3-5) failed to establish their structure.

We have postulated (6,7) similarities between the cyanogenetic principles in Kusum

[1]No Utiliz. Res. Dev. Div., ARS, USDA.

point between acetyl-CoA and fatty acids as in rats (18). Increased plasma free fatty acid levels have been observed (9) to precede decrease in hepatic fatty acid synthesis in the fasting chick. In *Salmo gairdnerii*, starving has also resulted in elevated blood free fatty acids (5). Free fatty acids may compete for coenzyme A, thereby limiting its availability for fatty acid synthesis. More work is needed to clarify these mechanisms in fish.

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Cyanolipids of Kusum (*Schleichera trijuga*) Seed Oil

ABSTRACT

A cyanolipid, consisting of two fatty acid moieties esterified with 1-cyano-2-hydroxymethylprop-2-ene-1-ol, constitutes 58% of *Schleichera trijuga* (Kusum) seed oil.

Oil obtained from the seeds of *Schleichera trijuga* (Kusum) is an item of commerce in India. A member of the Sapindaceae, *S. trijuga* is a large tree native to that general section of Asia. Cyanogenetic materials were detected in this oil some time ago (1,2); however, the latest reports concerning these interesting constituents (3-5) failed to establish their structure.

We have postulated (6,7) similarities between the cyanogenetic principles in Kusum

[1]No Utiliz. Res. Dev. Div., ARS, USDA.

oil and cyanolipids that we had identified from other (mainly sapindaceous) seed oils (6-10). To confirm (or deny) this hypothesis we isolated and identified the cyanolipids from a sample of *S. trijuga* seed procured from India.

Concurrent with our investigation, Vatakencherry (11 and personal communication) isolated a cyanolipid fraction from Kusum oil and demonstrated conclusively that it was identical to the diester cyanolipids we had isolated earlier from *Koelreuteria paniculata* seed oil (7). Our results confirm that these diesters occur in Kusum oil but only to the extent of about 6%. Acid-catalyzed methylation of this diester cyanolipid gave a methyl ester mixture having the following composition by gas liquid chromatography (GLC): $C_{12:0}$, 1.0%; $C_{14:0}$, 2.1%; $C_{16:0}$, 6.4%; $C_{16:1}$, 1.3%; $C_{18:0}$, 1.5%; $C_{18:1}$, 43.0%; $C_{18:2}$, 3.5%; $C_{20:0}$, 12.3%; $C_{20:1}$, 24.0%; $C_{20:2}$, 0.1%; $C_{22:0}$, 1.7%; and $C_{22:1}$, 3.1%.

We have also recovered another type of diester cyanolipid from Kusum oil in 58% yield by preparative thin layer chromatography (TLC). Silica Gel G layers 1 mm thick were developed in benzene to resolve these cyanolipids from ordinary triglycerides and the other cyanolipids (6%) present in the oil. The IR spectrum of these cyanolipids (after their recovery from preparative TLC) was superimposable on the spectrum of the cyanolipids first isolated from *Cordia* seed oil (6,8) and later from other seed oils (10). Similarly, the 100 MHz NMR spectrum of these Kusum oil cyanolipids (in $CDCl_3$) revealed proton counts, chemical shifts, multiplicities and coupling constants identical to those displayed by the *Cordia* nitriles (8). These data are conclusive evidence that the major cyanolipids of Kusum oil are diesters having two fatty acid moieties esterified with 1-cyano-2-hydroxymethylprop-2-ene-1-ol. GLC analysis of methyl esters derived by acid-catalyzed methylation of these cyanolipids gave: $C_{14:0}$, 1.6%; $C_{16:0}$, 4.6%; $C_{16:1}$, 1.0%; $C_{18:0}$, 0.9%; $C_{18:1}$, 32.8%; $C_{18:2}$, 2.7%; $C_{20:0}$, 29.0%; $C_{20:1}$, 22.1%; $C_{20:2}$, 0.1%; $C_{22:0}$, 2.1%; $C_{22:1}$, 3.1%.

Thus Kusum seed oil becomes the richest known source of this particular class of cyanolipids and it yields the largest total amount of cyanolipid material [64% as compared with 55% for *Cardiospermum halicacabum* seed oil (10)]. In fact, *S. trijuga* seed oil is similar to *Cardiospermum* oil because both contain the same two cyanolipid types in comparable quantities.

The reactivity of these cyanolipids, e.g., their conversion to lactones (4,6,11) and a dimethyl acetal (10,12) under specific conditions, as well as the instability of the free diol, has likely contributed to the uncertainty surrounding Kusum oil cyanolipids. Apparent failure to isolate glyceride-free cyano compounds in certain instances (3,5) has also made structural identification of the cyanolipids difficult. In addition, the published IR spectrum of Kusum oil (3,5) indicates free fatty acid (two carbonyl bands) and nitrile absorption. Conceivably, these bands could result from either seed variability or partial degradation of the oil because the IR spectrum of our oil sample exhibited neither free acid nor nitrile absorption. Only the minor cyanolipid fraction gives a nitrile band (weak) and 6% of this material in the oil is not enough to affect the IR spectrum of the oil.

Kasbekar and Bringi's (4) value of 37% of triglyceride in Kusum oil appears to confirm our value of 64% of total cyanolipids in this oil.

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Linoleic and Linolenic Acid as Precursors of the Cucumber Flavor

ABSTRACT

Cucumber homogenates were incubated with ^{14}C-linolenic and ^{14}C-linoleic acid. Of the radioactivity, 0.3% was recovered in the fraction of flavor active aldehydes. The distribution of the specific racioactivity indicated that propanal, *trans*-2-hexenal and *trans*-2,*cis*-6-nonadienal are related to 18:3 and hexanal and *trans*-2-nonenal to 18:2. A pathway for the development of these compounds is discussed.

The flavor of fresh cucumbers has been attributed largely to aldehydes. Forss et al. (1) have identified the pleasant element as *trans*-2,*cis*-6-nonadienal while 2-hexenal, 2-nonenal, ethanal, propanal and hexanal were considered to contribute secondarily to the overall flavor. Fleming et al. (2) had shown that the flavor is generated enzymatically when the fruit is cut or mechanically ruptured in the presence of oxygen.

The chemical structure of the aldehydes suggested that unsaturated fatty acids could be the precursors. The main fatty acids occurring in the lipids of cucumbers are linolenic (43%), palmitic (28%) and linoleic acid (20%) (W. Grosch and J.M. Schwarz, unpublished data). We incubated (U-^{14}C)-linolenic and (U^{14}C)-linoleic acid with a homogenate from cucumbers and analyzed the carbonyl compounds formed.

Green cucumbers approximately 1¼ in. in diameter were used. The source of labeled compounds were: (U^{14}C)-linolenic acid, (U^{14}C)-linoleic acid (specific activity 3200 μCi/mg, The Radiochem. Centre Amersham). Other materials were: linolenic acid and linoleic acid (98%), Roth; 2,4-dinitrophenylhydrazine (reagent grade, Merck), benzene and *n*-heptane free of carbonyl as described earlier (3), and aluminum oxide (Woelm, neutral, activity III). Deionized water was redistilled from a glass apparatus.

^{14}C-linolenic respectively ^{14}C-linoleic acid (500 mg, 10 μCi) was dissolved in a mixture of 0.4 ml Tween 20 and 20 ml water by dropwise addition of concentrated NaOH. After dilution with 80 ml water, the pH of the substrate was adjusted to 5.7 (pH of the cucumber sap) with concentrated HCl. Cucumbers (100 g) were cut into pieces and mixed (1-2 min) with the fatty acid emulsion in a waring blendor..

TABLE I

Aldehydes Formed From (U-^{14}C)-Linolenic and (U-^{14}C)-Linoleic Acid by a Cucumber Homogenate

Aldehyde	Precursor, Linolenic Acid		Precursor, Linoleic Acid	
	Mol-% Aldehyde	Specific Radio-activity[a]	Mol-% Aldehyde	Specific Radio-activity[a]
Ethanal[b]	14	21	5	12
Propanal[b]	46	41	3	3
Pentanal[b]	2	2	2	5
Hexanal[b]	3	2	47	48
Nonanal			4	7
Acroleine	3	1	1	1
Crotonaldehyde[b]	1	1	<1	1
2-Pentenal[b]	3	5	<1	1
2-Hexenal	9	7	1	1
2-Heptenal[b]			3	3
2-Octenal[b]			2	3
2-Nonenal	1	2	12	11
2,6-Nonadienal	16	11	9	1
2,4-Decadienal[b]			1	1
Unknown	2	7	10	2

[a]The measured radioactivity (cpm) was divided by the number of the C-atoms of the aldehyde. The sum of the specific activities of all compounds was taken as 100%.

[b]These aldehydes were also formed during the incubation of linolenic or linoleic acid with a crude preparation of cucumber lipoxidase (W. Grosch and J.M. Schwartz, unpublished data).

After incubation (60 min, room temperature), the mixture was extracted twice with 50 ml benzene. After adding 200 mg of 2,4-dinitrophenylhydrazine and 2 g of trichloroacetic acid, the combined extracts were heated for 1 hr at 60 C. The benzene solution was washed with water (acid free) and dried over Na_2SO_4. Aluminum (3 g) was added and the benzene was removed by evaporation in vacuo. The residue was put on the top of a 12 g aluminum column prepared in heptane. The monocarbonyl-2,4-dinitrophenylhydrazones (2,4-dnp) were eluted by benzene-heptane (1:1 v/v). The 2,4 dnp derivatives were separated, identified and quantitatively estimated as described in an earlier paper (3). The 2,4-dnp derivatives of unsaturated aldehydes were identified by thin layer chromatography according to Meijboom (4). The radioactivity was measured by a liquid scintillation spectrometer.

After incubation of a cucumber homogenate with ^{14}C-linolenic or ^{14}C-linoleic acid, approximately 0.3% of the radioactivity was recovered in the monocarbonyl fraction. With linolenic acid, 40 μmole aldehydes were isolated as 2,4-dnp and with linoleic acid 53 μmole. The distribution of the specific radioactivity in the 2,4-dnp (Table I) indicated that the aldehydes found by Forss et al. (1) in the cucumber flavor arise from unsaturated fatty acids. Hexanal and *trans*-2-nonenal are related to linoleic acid and propanal, *trans*-2-hexenal and *trans*-2,*cis*-6-nonadienal to linolenic acid. Also ethanal was formed mainly from this fatty acid.

A preliminary study of the pathway indiactes that a crude preparation of cucumber lipoxidase (EC 1.13.1.13) catalyzes the formation of the aldehydes which are marked in Table I. 2,6-Nonadienal, 2-nonenal and 2-hexenal were formed only in very small amounts. For this reason and since the production of aldehydes in the cucumber homogenate is relatively high while the lipoxidase content in this tissue is small (5), we suggest that another pathway of aldehyde formation dominates in this vegetable. We therefore propose that in cucumber the double bonds of the unsaturated fatty acids are broken in a dioxygenase-like reaction. Such a reaction would lead to the formation of hexanal and *cis*-3-nonenal from linoleic acid while propanal, *cis*-3-hexenal and *cis*-3,*cis*-6-nonadienal would be formed from linolenic acid. The isomerization of the 3-*cis* double bond to the 2-*trans* in the aldehydes may occur after the enzyme catalyzed oxidation of the unsaturated fatty acids has taken place.

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Phospholipid Composition of the Boll Weevil, *Anthonomus grandis* Boheman

ABSTRACT

When phospholipids of newly-emerged adults of the boll weevil, *Anthonomus grandis* Boheman, were studied in detail, phosphatidyl choline and phosphatidyl ethanolamine were found to be the major phospholipids; sphingomyelin and cardiolipin were present in smaller amounts, and four other minor components were identified. Fatty acid analyses performed on the intact phospholipids and on the enzyme degradation products of phosphatidyl choline and phosphatidyl ethanolamine demonstrated that oleic and linoleic acids were the major fatty acids present in the glycerophosphatides; the sphingomyelin contained fatty acids in the range of 20-22 carbons.

After incubation (60 min, room temperature), the mixture was extracted twice with 50 ml benzene. After adding 200 mg of 2,4-dinitrophenylhydrazine and 2 g of trichloroacetic acid, the combined extracts were heated for 1 hr at 60 C. The benzene solution was washed with water (acid free) and dried over Na_2SO_4. Aluminum (3 g) was added and the benzene was removed by evaporation in vacuo. The residue was put on the top of a 12 g aluminum column prepared in heptane. The monocarbonyl-2,4-dinitrophenylhydrazones (2,4-dnp) were eluted by benzene-heptane (1:1 v/v). The 2,4 dnp derivatives were separated, identified and quantitatively estimated as described in an earlier paper (3). The 2,4-dnp derivatives of unsaturated aldehydes were identified by thin layer chromatography according to Meijboom (4). The radioactivity was measured by a liquid scintillation spectrometer.

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TABLE I

Phospholipid Content of the Boll Weevil

Phospholipid	Percentage of total phospholipid
Lysophosphatidyl choline	2.5
Sphingomyelin	7.5
Lysophosphatidyl ethanolamine	1.8
Phosphatidyl choline	35.5
Phosphatidyl inositol	5.2
Phosphatidyl serine	5.1
Unknown	1.2
Phosphatidyl ethanolamine	32.7
Cardiolipin	8.0

Previous reports on the lipids of the boll weevil, *Anthonomus grandis* Boheman, have dealt with the identification of total fatty acids (1) and the synthesis and storage of fatty acids (2,3). Work with the phospholipids has been confined to a study of the alkyl and alkyl-1-enyl glyceryl ethers of plasmalogens (4). The present communication describes the isolation and detailed characterization of the phospholipid components.

The insects used in the tests were reared at the Boll Weevil Research Laboratory at State College, Miss., on the modified larval diet of Gast and Davich (5). Newly emerged unfed adults were collected and extracted with chloroform-methanol (2:1), and the extracts were washed according to Folch et al. (6). The extracted lipids were dried over sodium sulfate and concentrated on a rotary evaporator.

The total lipids were separated into neutral and phospholipid classes by column chromatography on silicic acid by eluting first with chloroform and then methanol. Then the phospholipids were separated by two-dimensional thin layer chromatography (TLC) in systems developed by Abramson and Belcher (7) and Rouser et al. (8). The TLC separations were performed on standard 20 x 20 cm glass plates coated with 250 μ layers of silica gel-calcium sulfate (9:1 w/w). Large quantities of the individual phospholipids were prepared by using a combination of silicic acid column chromatography, DEAE cellulose chromatography (9) and TLC.

Fatty acid methyl esters of phosphatides were prepared with boron trifluoride-methanol by the method of Metcalfe et al. (10); those of sphingomyelin were prepared in a sealed tube with 10% methanolic H_2SO_4 at 100 C for 4 hr. Qualitative and quantitative determinations of the methyl esters were made by GLC equipped with a flame ionization detector on a 6 ft, 1/8 o.d. column packed with 10.5% diethylene glycol adipate on 60/80 mesh hexamethyldisilazane-treated gas chrom P. The column temperature was 190 C. The instrument was calibrated with authentic standard mixtures of fatty acid methyl esters. Quantification was obtained by peak triangulation.

Phosphorus was determined by the method of Chen et al. (11) as modified by Mitlin (12). Amino nitrogen analyses were performed according to the procedure described by Lea and Thodes (13).

Lyophilized venom of the eastern diamondback rattlesnake, *Crotalus adamanteus*, was used for the selective hydrolysis of phosphatidyl choline; the venom of the cotton-mouth moccasin, *Ancistrodon piscivorus piscivorus*, was used for the hydrolysis of phosphatidyl ethanolamine. The conditions used for the hydrolyses and subsequent isolation of the fatty acids were those described by Menzel and Olcott (14).

The average amount of total lipid isolated was 2.4% of the fresh weight of the weevils. Of the total lipid, 59.2% was phospholipid; the remainder was neutral lipid.

The individual phospholipids were identified on the basis of their chromatographic properties and their reactivity to spray reagents (15). Co-chromatography with authentic standard

TABLE II

Fatty Acid Composition of the Major Phospholipids of the Boll Weevil

	Fatty acids, as mole per cent of total fatty acid									
Phospholipid[a]	14:0	14:1	16:0	16:1	18:0	18:1	18:2	18:3	20:1	22:0
PC	0.2	—	7.9	10.7	4.4	46.2	28.7	0.9	—	—
PE	0.1	—	8.1	8.0	9.4	41.7	29.3	3.1	—	—
PGP	0.8	0.6	5.6	15.8	1.4	18.0	53.0	4.3	—	—
Sph	0.3	—	2.0	0.7	4.8	2.2	—	—	58.4	31.4
LPC	0.5	—	12.7	10.2	12.8	36.6	26.9	—	—	—
LPE	—	—	20.6	4.1	28.0	36.2	10.9	—	—	—

[a]PC, phosphatidyl choline; PE, phosphatidyl ethanolamine; PGP, polyglycerophosphatide; Sph, sphingomyelin; LPC, lysophosphatidyl choline; LPE, lysophosphatidyl ethanolamine.

TABLE III

Positional Distribution of Fatty Acids of Boll Weevil Phosphatidyl Choline and Phosphatidyl Ethanolamine as mole per cent of total fatty acids

Fatty acid carbon No.	Phosphatidyl choline		Phosphatidyl ethanolamine	
	Position		Position	
	1	2	1	2
14:0	0.5	0.3	Trace	Trace
16:0	12.7	5.5	20.6	10.3
16:1	10.2	8.6	4.1	10.8
18:0	12.8	—	28.0	—
18:1	36.6	62.0	36.2	61.7
18:2	26.9	22.0	10.9	16.9
18:3	—	1.4	Trace	Trace
Unsaturated	73.8	94.1	51.2	89.7
Saturated	26.2	5.9	48.8	10.3

phospholipids and chemical analysis of fatty acids, phosphorus and amino nitrogen were also used for identification. The phospholipids were quantitated by separation in two-dimensional TLC, charring the spots with sulfuric acid reagent, scraping the spots from the plates and analyzing for phosphorus. At least three of these analyses were performed on each compound.

The composition and percentage distribution of the phospholipid mixture in the boll weevil is shown in Table I. As in other Coleoptera (16), phosphatidyl choline and phosphatidyl ethanolamine were the most abundant phospholipids, and their percentages were about equal.

The fatty acid composition of the major phospholipids is given in Table II. In both phosphatidyl choline and phosphatidyl ethanolamine, over 80% of the acids were unsaturated, and oleic and linoleic acids comprised over 70% of the total acids. These figures are significantly different from those obtained for other Coleoptera in which the oleic acid content of phosphatidyl choline and phosphatidyl ethanolamine ranges from 20% to 30% (16). Also, the presence of cardiolipin is unique because it has a high linoleic acid content (53%), but this acid is the major one in the diet of the larval boll weevil so it is probably involved in the active transport and metabolism of linoleic acid. The sphingomyelin contained unusually large amounts of 20 and 22 carbon acids and $C_{20:1}$ and $C_{22:0}$ comprised about 90% of the total. The presence of these acids was confirmed by direct comparison with authentic standards and by hydrogenation, then comparison with saturated standards.

Table III shows the results of the enzymatic hydrolyses of phosphatidyl choline and phosphatidyl ethanolamine. The 2 position of both compounds contained large percentages of unsaturated fatty acids; the major one (62%) was oleic acid. The 1 position also had an abundance of unsaturated fatty acids, but large increases were observed in the percentage of saturated fatty acid. In both compounds, stearic acid was not observed in the 2 position. The positional distribution of fatty acids in phosphatidyl choline and phosphatidyl ethanolamine was therefore similar to that found in animals, but no comparison with other insects can be made because we lack the necessary data. Also, the determination of the fatty acid composition of cardiolipin and sphingomyelin is noteworthy since no comparable information is available for other insects.

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Improved Separation of Phospholipids by Counter-Current Distribution

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Separations by counter-current distribution of individual molecular species of phospholipids are much improved by the use of CCl_4:CH_3OH:H_2O (62:33:5 v/v) for lecithins and of heptane:CH_3OH:H_2O (50:47.5:2.5 v/v) for the methyl esters of the dinitrophenyl derivatives of phosphatidylethanolamines.

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The solvent system first employed for phosphatidylcholine was that devised by Cole, Lathe and Ruthven (5) and consisted of CCl_4-methanol-water (62:33:3.15 v/v). In the present investigation the content of carbon tetrachloride was kept constant at 62% by volume while the water content was varied between 3.15% and 7.0% by volume. An antioxidant (10 mg/liter of 2:6-di *tert* butyl-4-methyl phenol) was added and a small sample

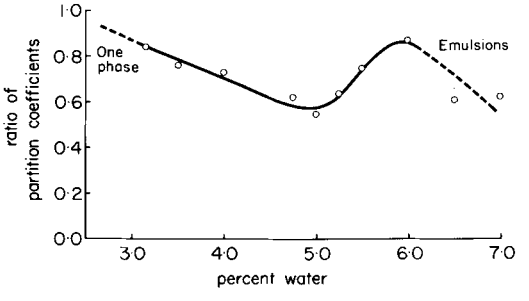
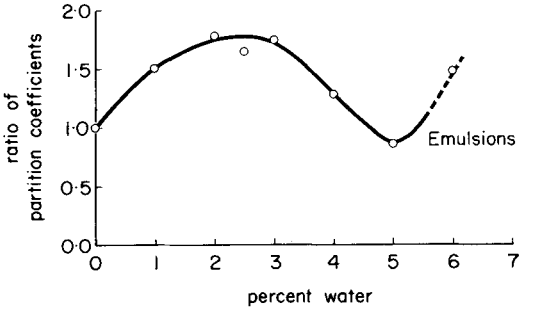


FIG. 1. Using the solvent system CCl_4:CH_3OH:H_2O (62:38 w/w, by volume), where w represents the per cent water, phosphatidylcholine was equilibrated between equal volumes of upper and lower phases and the proportion of fatty acids measured by gas chromatography in each phase. The ratio of stearic acid to palmitic acid was recorded for each phase and the ratio of these ratios plotted as shown. Maximal separation was obtained when w = 5.0.



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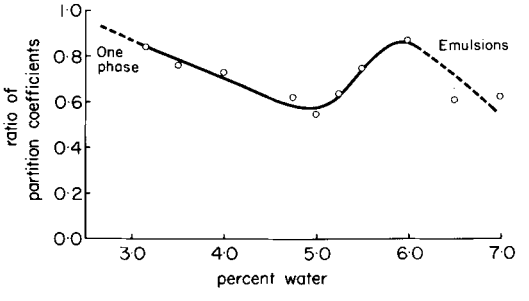

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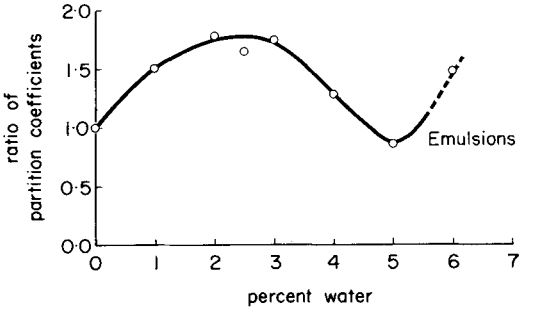

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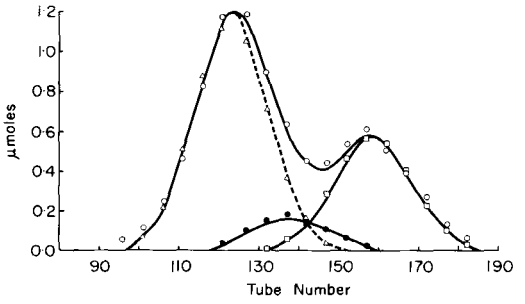

FIG. 3. The counter-current distribution for 400 transfers in CCl_4:CH_3OH:H_2O (62:33:5 v/v) of a mixture of 1-stearoyl-2-arachidonoyl-, 1-oleoyl-2-arachidonoyl- and 1-palmitoyl-2-arachidonoyl-phosphatidylcholine.

(0.3 to 0.5 mg) of phosphatidylcholine added to equal volumes (10 ml) of previously equilibrated upper and lower phases obtained from the solvent mixtures. An approximate value of the ratio of the partition coefficients of the stearoyl compounds to the palmitoyl compounds could be obtained by dividing the ratio of stearic acid to palmitic acid in the top phase by the corresponding ratio in the bottom phase. The fatty acids were measured by gas chromatography (6). This double ratio gave an approximate measure of the separation due to two carbon atoms. The results for phosphatidylcholine in the system CCl_4-methanol-water are shown in Figure 1. As will be seen, the maximum separation was obtained in the solvent system CCl_4-methanol-water (62:33:5 v/v).

Figure 2 shows the results obtained with a sample of phosphatidylcholine containing only stearic, oleic, palmitic and arachidonic acids. The partition coefficients of stearoyl-arachidonoyl-, oleoyl-arachidonoyl- and palmitoyl-arachidonoyl-phosphatidylcholines were respectively 0.446, 0.528 and 0.660.

Figure 3 shows the effect of altering the water content in the system heptane-methanol-water on the separation of the methyl esters of dinitrophenylated phosphatidylethanolamines differing by two carbon atoms and the best separation was obtained with the system heptane-methanol-water (50:47:5:2.5 v/v). Counter-current distribution of a sample containing arachidonic acid as the only polyenoic fatty acid showed that the partition coefficients of stearoyl arachidonoyl, oleoyl arachidonoyl and palmitoyl arachidonoyl phosphatidylethanolamines (methylesters of the DNP-derivatives) were respectively 1.31, 0.950 and 0.877.

These changes in the solvent system would enable stearoyl arachidonoyl phosphatidylcholine to be separated from palmitoyl arachidonoyl phosphatidylcholine with 860 transfers instead of 3300 transfers. The corresponding improvement in the separation of phosphatidylethanolamine would be 750 transfers instead of 1530 transfers. This technique of counter-current distribution has been used by Collins (1) and Trewhella and Collins (7) to enable the specific radioactivities of phospholipids species to be calculated even though a complete separation was not possible. The improved separation now obtainable will ensure increased accuracy.

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The Functioning of the Lipids and Lipoproteins of Sarcotubular Membranes in Calcium Transport[1]

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ABSTRACT

In the intact muscle cell, an internal tubular membrane system called the sarcoplasmic reticulum (SR) plays an important role in the contraction-relaxation cycle by controlling the Ca^{++} of the myoplasm; release of Ca^{++} from the SR to myoplasm initiates contractile activity and sequestring Ca^{++} in the SR by means of a transport system causes muscle to relax. Fragments of the SR with a vesicular structure can be isolated from muscle homogenate and these vesicles are able to vigorously transport Ca^{++} from incubation media into the intravesicular space thus enabling study of Ca^{++} transport under precisely defined in vitro conditions. A highly purified fraction of SR vesicles called SF_1 were prepared from rat muscle by means of density gradient centrifugation procedures. The role of SR lipid in Ca^{++} transport was studied. SF_1 was treated in vitro with either phospholipase A or C or D or polyene antibiotics. The effect of essential fatty acid deficiency, induced in vivo, was also investigated. It was concluded that the only structural feature of SF_1-lipid involved in Ca^{++} transport and the associated adenosine triphosphatase is the phosphoryl moiety of the phospholipids. Evidence was obtained which inplicated histidine residues of the SF_1 protein in this transport function. To study the role of SF_1 protein in this process in depth, the membranes were solubilized by a sodium dodecylsulfate system and made free of their lipid components. More than 95% of this protein is soluble in dilute salt solution; of this, more than 90% is composed of a protein fraction which can be isolated by gel filtration (called protein fraction-2). Protein fraction-2 contains large molecular aggregates of small polypeptide subunits of identical or nearly identical molecular weight. They contain solely N-terminal glycine and probably only C-terminal alanine. The significance of such a high percentage of similar polypeptide subunits in SR is discussed.

The molecular structure of biological membranes has been the subject of much investigation in recent years (1-8). These studies have established lipid and protein as the major components of membranes and have also shown that many different protein species are present in the membranes so far studied. The chemical complexity of membranes, particularly that caused by multiplicity of proteins, has made it difficult to study readily the detailed molecular structure and its relationship to functional activity. Obviously a membrane with a relatively simple protein composition would facilitate such study by providing an experimental prototype of much less molecular complexity.

A review of the literature suggested that the sarcotubular membranes of skeletal muscle might well provide this prototypic membrane. These membranes, the morphology of which is schematically (9) shown in Figure 1, function in vivo in the processes of excitation-contraction coupling and muscle relaxation, phenomena accomplished, respectively, by the releasing Ca^{++} to the myoplasm and sequestering Ca^{++} from the myoplasm (10). The Ca^{++}-sequestering function has been studied with in vitro systems utilizing vesicular sarcotubular membrane fragments isolated by differential centrifugation; these vesicles vigorously take up Ca^{++} from incubation media if ATP is available (11), some of the Ca^{++} being bound to the membrane structure and some concentrated intravesicularly. On the basis of data obtained in his and in Martonosi' laboratory, Mommaerts (12) theorizes that there is little room in the sarcotubular membrane for protein that is not actively involved in this Ca^{++} transport process. If this theoretically based concept of Mommaerts is actually the case, it seems likely that sarcotubular membranes are composed of a quite limited number of protein species and are therefore the kind of prototypic membrane we seek.

Therefore, we decided to study the sarco-

[1]One of five papers to be published from the Symposium "Lipid Transport" presented at the AOCS Meeting, New Orleans, April 1970.

tubular membranes of rat skeletal muscle and as a first step developed a method for the isolation of membranes which are primarily, if not exclusively, derived from sarcoplasmic reticulum. This was achieved by combining differential centrifugation with the sequential use of two density gradient centrifugation steps (13), the flowsheet for the method is presented in Figure 2. Procedures for preparing the sucrose density gradients are summarized in Figure 3. The following is a brief description of the procedure. Rat skeletal muscle is homogenized in 0.3 M sucrose by blending for 30 sec in a Waring blender at full speed. The homogenate is centrifuged at 15,500 x *g* for 20 min in rotor SS 34 in a Servall centrifuge and the supernatant collected and filtered through gauze. The filtered supernatant is centrifuged for 90 min at 56,500 x *g* in rotor No. 40 in Spinco Model L centrifuge. The pellet from this step, designated F_o, is a crude microsome preparation. Fraction F_o, suspended in 0.3 M sucrose, is layered on top of sucrose gradient system I and centrifuged at 90,000 x *g* for 2 hr in swinging bucket rotor (SW 25.1) in a model L Spinco to yield two subfractions, F_1 and F_2 (Fig. 2 and 3). The F_1 subfraction is suspended in 0.25 M sucrose and further subfractionated by layering on top of sucrose gradient II and centrifuging at 57,600 x *g* (SW 25.1) for 50 min to yield two subfractions designated SF_1 and SF_2 (Fig. 2 and 3).

The relative ability of the subfractions to take up Ca^{++} in the presence of ATP was com-

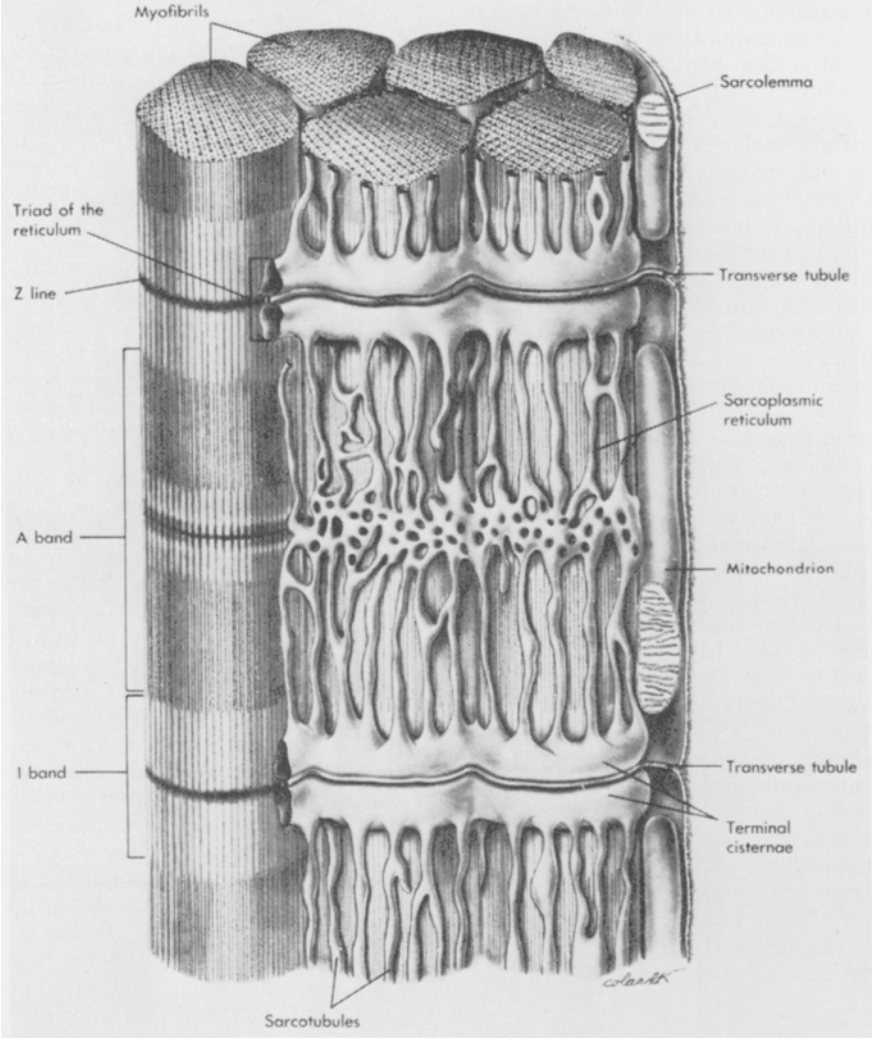

FIG. 1. Schematic representation of sarcotubular membranes of skeletal muscle. [From Bloom and Fawcett (9)].

pared (Fig. 4); the technique for measuring Ca^{++} uptake has been described (13). SF_1 has the greatest ability to take up Ca^{++} per milligram of protein. The Mg adenosine triphosphatase specific activities of each subfraction was measured as previously described (13); subsequently it was found that the Mg adenosine triphosphatase is almost completely suppressed by EGTA and that this inhibition of the adenosine triphosphatase can be reversed by addition of Ca^{++}. Therefore the adenosine triphosphatase activity reported in Figure 5 relates primarily to the (Ca^{++} + Mg^{++})-adenosine triphosphatase that has been linked to Ca^{++} transport activity. Subfraction SF_1 has the highest specific activity. Electron microscopic examination of SF_1 reveals it to be composed primarily of membrane vesicles in contrast to subfraction SF_2 which contains nonvesicular structures as well as mitochondria (Fig. 6). On the basis of morphological appearance, Ca^{++} transport and the associated adenosine triphosphatase activity, stability of the Ca^{++} transport activity upon storage at 2 C, we felt that SF_1 was a sufficiently purified population of sarcotubular membranes to permit exploration of its molecular properties. A recent morphological analysis of the membranes by Deamer and Baskin (14) supports this conclusion.

Our first approach was to study the role of lipids in the Ca^{++} transport activity. Although these membranes contain small quantities of cholesterol and other neutral lipids, phospholipid accounts for most of the lipid. The membranes of fraction SF_1 contained a remarkably constant amount of phospholipid relative to the amount of protein, the average value being 0.611 μEq of lipid P per milligram of protein

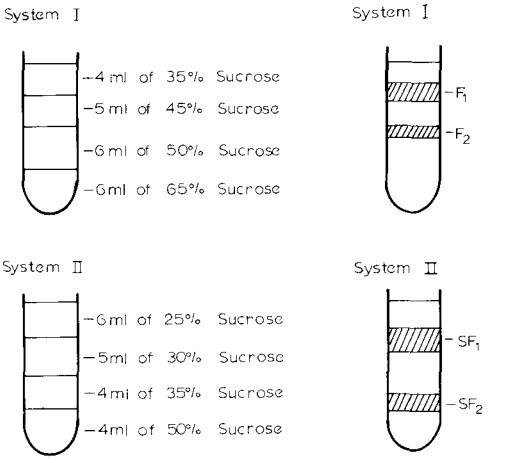

FIG. 3. Preparation of sucrose density gradients: System I involves layering the sucrose solutions in the volumes and concentrations noted in the diagram at 20 C and them letting the system stand at 2 C for 17 hr before using; system II involves layering the sucrose solutions in the volumes and concentrations noted in the diagram at 20 C before using. The tubes to the right in the diagram show the distribution of microsomal subfractions in sucrose density gradients after centrifugation. The symbols used to designate the subfractions are noted on the diagrams [From Yu et al. (13)].

(15). Choline-containing phospholipid accounts for 70% of this lipid-P, phosphatidyl-choline being overwhelmingly the major phospholipid class present.

Martonosi (16,17) reported that treatment of muscle microsomes with phospholipase C caused hydrolysis of much of the membrane lecithin and at the same time caused a loss in the ability of the microsomes to actively take

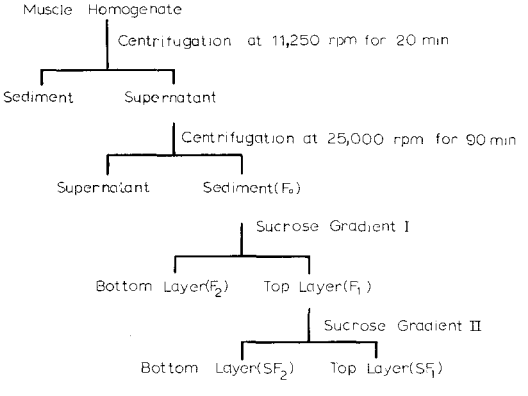

FIG. 2. Flowsheet for fractionation of muscle homogenates and muscle microsomes [From Yu et al. (13)].

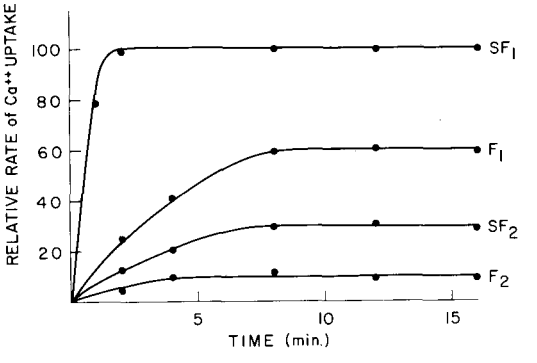

FIG. 4. Comparison of Ca^{++} uptake by various microsomal subfractions [From Yu et al. (13)]; the ordinate is expressed in terms of Ca^{++} uptake activity on a per 100 μg protein basis relative to that of fraction SF_1 incubated for 16 min.

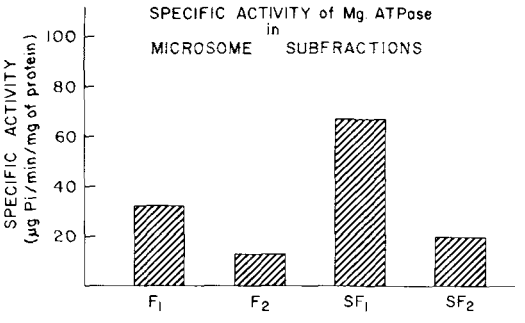

FIG. 5. Specific activity of Mg-adenosine triphosphatase in microsome subfractions [From Yu et al. (13)].

up Ca^{++} and a loss of the associated adenosine triphosphatase activity. We have confirmed these results with fraction SF_1 (Fig. 7).

However, treatment of SF_1 with phospholipase D had no effect on sarcotubular Ca^{++} uptake, even after 70% of the choline-containing phospholipids were caused to hydrolyze to phosphatidic acid and choline (Fig. 8). Phospholipase D treatment caused a small but significant increase in Mg-adenosine triphosphatase activity.

Treatment with phospholipase A resulted in hydrolysis of 50% of the fatty acid in ester linkage in the 2 position of the phosphoglycerides of sarcotubular membranes but caused little loss in the amount or rate at which Ca^{++} could be taken up by the sarcotubular membranes (Fig. 9). The small decrease in Ca^{++} uptake noted in the Figure probably is due to an increase in membrane FFA since FFA are potent inhibitors of this Ca^{++} transport system (18). In the experiment reported in Figure 9, 2% albumin (fatty acid-poor) was added to the media; if phospholipase A treatment is carried out in absence of albumin, Ca^{++} uptake is rapidly lost, presumably because, in this case, most of the FFA released from phosphoglycerides during phospholipase A treatment remains with the membrane rather than being trapped by the albumin.

The role of the fatty acid moieties in the sarcotubular membrane lipids in the Ca^{++} transport system was further evaluated by comparing this activity in membranes derived from normal and essential fatty acid deficient rats. The membranes of essential fatty acid deficient rats had a very different spectrum of fatty acids than the normal animals (Table I), they contained much less stearic, linoleic and arachidonic acids and much more oleic and eicosatrienoic acids than the vesicles from normal rats. Nevertheless, neither the amount of Ca^{++} uptake by the vesicles nor the rate of uptake was depressed by this marked change from the normal pattern of fatty acids in the sarcotubular membrane lipids (Fig. 10); indeed there may even be some increase in rate of Ca^{++}

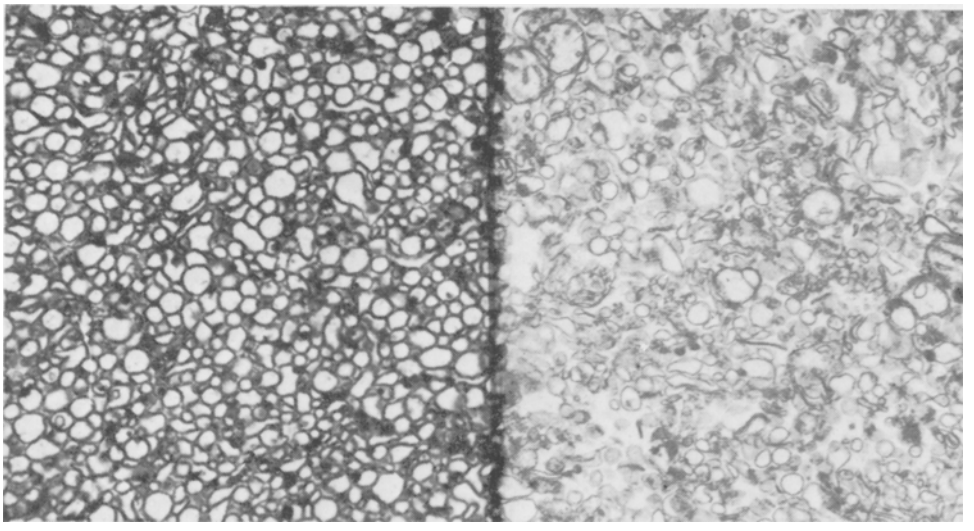

FIG. 6. Electron micrographs of subfractions SF_1 (left) and SF_2 (right). The pellet of each subfraction is fixed with 1% OsO_4 in 0.065 M collidine buffer, pH 7.0, embedded in Epon 812 and stained with lead citrate. Magnification 32,000X [From Yu et al (13)].

TABLE I

Effect of Essential Fatty Acid Deficiency on Fatty Acid Composition of Sarcoplasmic Reticulum[a,b]

Fatty acid	Control[c]	EFA-Def.[b]
16:0[d]	31 ± 1.2	28 ± 2.0
16:1	9 ± 1.6	13 ± 1.9
18:0	19 ± 0.8	5 ± 0.5
18:1	4 ± 1.1	39 ± 2.3
18:2	18 ± 1.7	1 ± 0.9
20:3	1 ± 0.6	10 ± 1.8
20:4	10 ± 2.1	2 ± 0.8

[a]Lipids were extracted from sarcotubular vesicles pooled from two to five rats; five such pools were analyzed both for control rats and for essential fatty acid-deficient (EFA-Def.) rats.

[b]From Yu et al. (15).

[c]Moles % ± S.D.

[d]Fatty acids are designated by number of carbon atoms:number of double bonds.

uptake in membranes from deficient rats.

Polyene antibiotics interact with sterols of biological membranes, causing reorientation of membrane lipids (19). Although sarcotubular membranes contain only small amounts of cholesterol, the effect of these antibiotics on Ca^{++} uptake was investigated. The sarcotubular membranes bound about 0.25 μmoles of filipin per milligram of sarcotubular protein but the binding of this antibiotic did not influence either Ca^{++} uptake or Mg-adenosine triphosphatase activities (Fig. 11). Treatment with nystatin and pimaricin was also without effect.

The striking aspect of these studies on sarcotubular lipids is the lack of sensitivity of the Ca^{++} transport system to very marked alterations in lipid structure. Even with phospholipase C, the only treatment to inhibit Ca^{++} transport, a large destruction of membrane phospholipid was needed; indeed a greater than 50% hydrolysis of the phosphodiester bonds of phospholipids had to occur before significant loss of Ca^{++} transport was observed.

These results led us to the focus on the protein components of these membranes. It was shown by Hasselbach and Seraydarian (20) in 1966 that certain sulfhydryl groups in these membranes, presumably from cysteine residues of membrane protein, are involved in the Ca^{++} transport process. Moreover preliminary work (21) in our laboratory implicated the functioning of imidazole groups, presumably from histidine residues of the membrane protein, in the Ca^{++} uptake system. However before extensively studying the role of functional groups of sarcotubular membrane proteins in the Ca^{++} transport process, we decided to learn

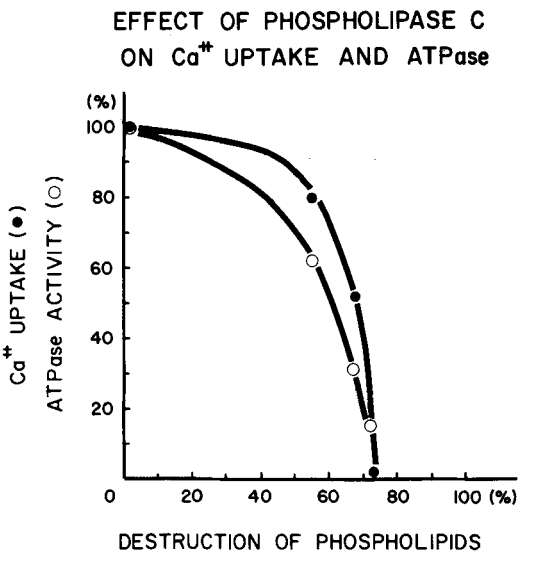

FIG. 7. Effect of phospholipase C on Ca^{++} uptake and Mg^{++}-adenosine triphosphatase of sarcotubular membranes. The Y axis refers wither to the maximum uptake of Ca^{++} (closed circles) by phospholipase-treated sarcotubular membranes as a percentage of the maximal uptake observed with untreated sarcotubular membranes processed identically or to the Mg^{++}-adenosine triphosphatase (open circles) expressed in the same way. The X axis refers to the percentage hydrolysis of phospholipid [From Yu et al. (15)].

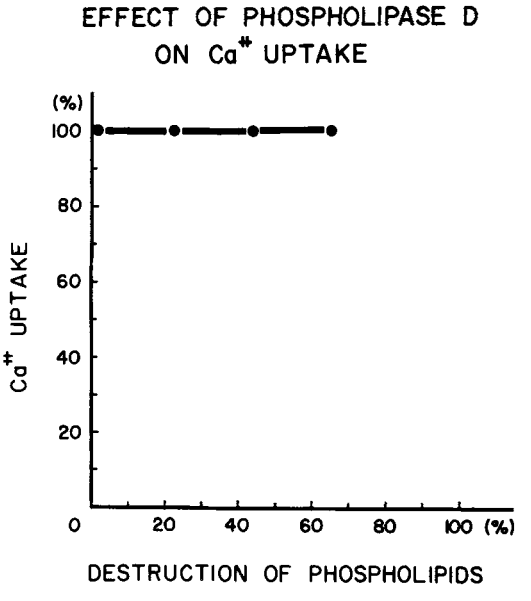

FIG. 8. Effect of phospholipase D on Ca^{++} uptake by sarcotubular membranes. For explanation of Y axis see Figure 7. The X axis refers to percentage hydrolysis of choline-containing phospholipid [From Yu et al. (15)].

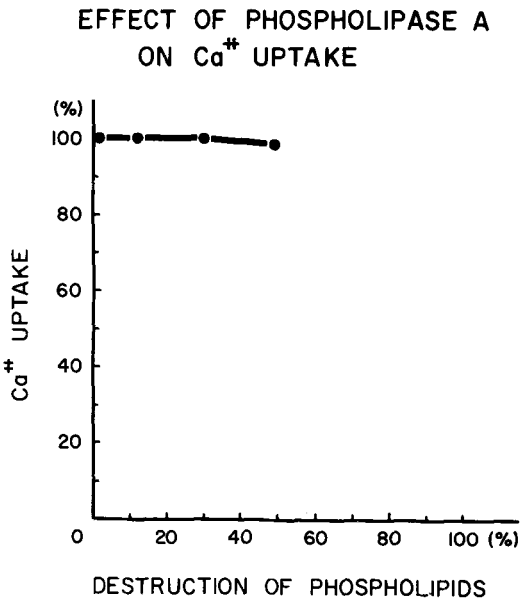

FIG. 9. Effect of phospholipase A on Ca^{++} uptake by sarcotubular membranes. For explanation of X and Y axes see Figure 7 [From Yu et al. (15)].

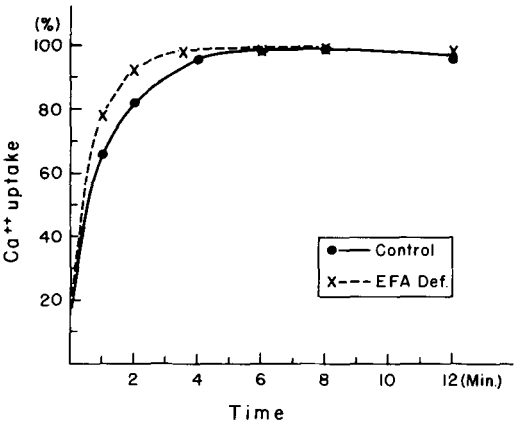

FIG. 10. Comparison of the uptake of Ca^{++} by sarcotubular membranes from normal and essential fatty acid deficient (EFA-Def) rats. The Y axis refers to percentage of 0.4 μmoles of Ca^{++} taken up by the sarcotubular membranes. Closed circles designate "normal" sarcotubular membranes and the X' designate "essential fatty acid deficient" sarcotubular membranes [From Yu et al. (15)].

more about the protein structure of these membranes.

For such an investigation, it is necessary to solubilize the sarcotubular membrane proteins. We first tried sonic treatment of the membranes since Barclay et al. (22) reported in 1967 that rat liver plasma membranes were solubilized by a mild sonic treatment which yielded three classes of high density lipoproteins and one class of protein. Sonic treatment of the milky-appearing suspension of sarcotubular membranes did lead to a transparent-looking preparation but electron microscopy revealed this sonicated material to be composed of finely divided membrane fragments. Delipidation of the membranes followed by succinylation (23) of the protein suspended in 8 M urea or a direct attempt to dissolve the protein in 0.9% formic acid failed to dissolve significant amounts of protein. Other attempts were made to solubilize the sarcotubular membrane protein but only the method of dissolving the sarcotubular membranes in a low ionic strength solution containing sodium dodecylsulfate (SDS) which we recently described was successful (24). After such treatment the protein remains soluble even after most of the SDS is removed by extensive dialysis provided a low ionic strength salt solution is used instead of distilled H_2O.

Analysis of this solubilized protein by Sephadex G-200 column chromatography yielded two protein fractions, one with a V_e (elution volume) equal to the V_o (void volume) and another with a $V_e > V_o$. Most of the protein is in the latter fraction which has a V_e/V_o ratio of 1.27. However the resolution of these two fractions was poor. In an attempt to improve the resolution, Sepharose 4B gel filtration was used. Again two protein fractions emerged, one at the V_o and one sufficiently after the V_o to permit good resolution. The second fraction, fraction-2, contains more than 90% of the protein applied to the column (Fig. 12). Fraction-2 contains at most only traces of SDS (SDS/protein weight ratio < 0.001). This protein has little lipid with a phospholipid-protein weight ratio of 0.05 compared to 0.49 for the intact sarcotubular membranes.

The contents of the tubes containing fraction-2 were combined to yield a first (tubes 35-40), second (tubes 41-45) and third (tubes 46-50) subfraction of it relative to the order of elution from the Sepharose 4B. Data on the amino acid composition of each subfraction are presented in Table II. The amino acid composition of each subfraction was similar; any differences noted were within the error of the analytic procedures.

Cellulose acetate strip electrophoresis was used for the analysis of fraction-2 in the pH range of 6.5 to 9.5. The results at pH 7.0, 7.6, 8.5 and 9.3 are presented in Figure 13 (25). At pH 6.5 the protein did not migrate while at the higher pH values it migrated in a single band towards the anode.

The protein in fraction-2 did not electro-

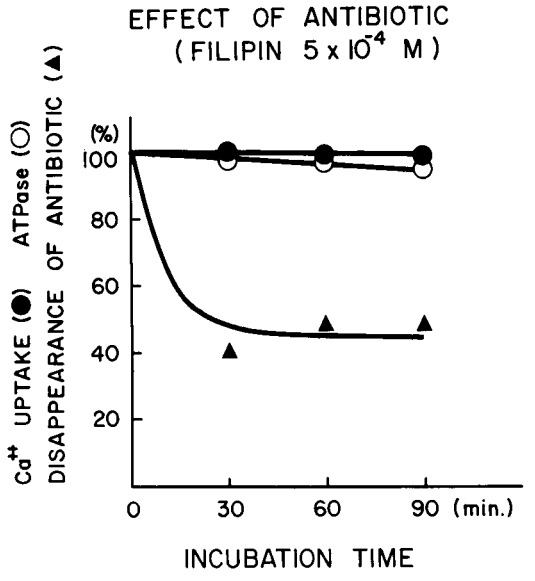

FIG. 11. Effect of filipin on Ca^{++} uptake by sarcotubular membranes. The Y axis refers either to maximum uptake of Ca^{++} by filipin-treated sarcotubular membranes in terms of percentage of the maximal uptake observed with untreated sarcotubular membranes processed identically (closed circles) or to Mg^{++}-adenosine triphosphatase activity expressed in the same way (open circles) or to the percentage of added filipin taken up by sarcotubular membranes (solid triangles). The X axis refers to the time of incubation with filipin [From Yu et al. (15)].

TABLE II

Amino Acid Composition of Subfractions of Fraction-2[a,b,c]

Amino acid	Subfractions 1st	2nd	3rd
Lysine	111	123	118
Histidine	38	43	41
Arginine	123	125	116
Aspartic Acid	303	301	292
Threonine	143	153	124
Serine	191	184	215
Glutamic Acid	290	355	219
Proline	140	152	122
Glycine	206	205	179
Alanine	207	223	196
Valine	130	143	126
Isoleucine	89	98	90
Leucine	265	269	271
Tyrosine	50	49	45
Phenylalanine	83	105	102
Cysteic Acid	10	10	10

[a]Expressed in μmoles/100 mg of fraction-2. Tryptophan and methione were not measured.

[b]To determine the amino acid content of a given protein fraction, it is hydrolyzed in evacuated tubes in 6 N HCl at 110 C for 24 hr. After hydrolysis, the HCl is evaporated and the dried sample is then dissolved in 0.2 M citrate buffer (pH 2.2). Amino acid analysis is performed with a Phoenix (Model M-7800) amino acid analyzer. The columns were packed with Phoenix Spherix type XX8-10-0 for short columns and XX8-60-0 for long columns. Cysteic acid analysis is carried out by the method of Moore (32).

[c]From Yu and Masoro (25).

phoretically enter polyacrylamide gels at 4% or higher concentrations. Since gels with less concentration than 4% were impossible to handle in the vertical gel electrophoresis apparatus available, a 3% polyacrylamide-0.5% agarose gel system was used. The protein of fraction-2 enters this gel (which can be easily handled) and migrates in two broad bands (Fig. 14).

Analysis of fraction-z by analytical ultracentrifugation in the presence and absence of 2-mercaptoethanol was carried out. In the presence of 2-mercaptoethanol at least three components were observed (Fig. 15a); the major one had a S_{20} value of 6.5. In the absence of 2-mercaptoethanol the fast moving minor component was not detected (Fig. 15b).

The data presented to this point clearly show that the protein in fraction-2 is of high molecular weight. On the basis of the likelihood that these high molecular weight structures are aggregates of smaller protein components, an attempt was made to dissociate the protein in fraction-2 by treating it with SDS and 2-mercaptoethanol according to the method of Shapiro et al. (26). Analysis of the dissociated protein electrophoretically in a 5% polyacrylamide gel containing 0.1% SDS revealed that all protein entered the gel and most of it migrated in a single band (Fig. 16). Estimation of molecular weight of this dissociated protein on the basis of migration distance relative to migration of standard proteins as described by Shapiro et al. indicated a molecular weight of

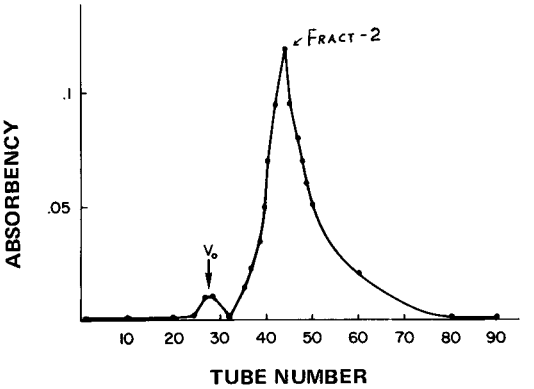

FIG. 12. Sepharose 4B gel filtration analysis of solubilized proteins from SF_1 [From Masoro and Yu (24)].

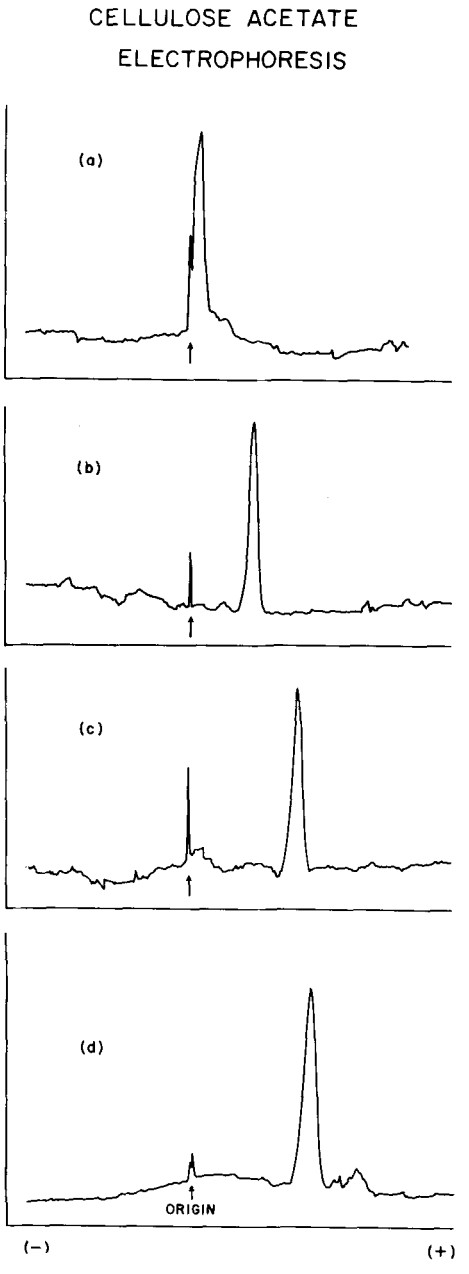

FIG. 13. Cellulose acetate strip electrophoretic analysis of fraction-2. The Millipore phoroslide system was used; the buffer was sodium barbital-HCl (0.05 M). The electrophoresis was carried out under the following conditions: temperature, 23 C; time, ½ hr; voltage, 100 V. The strip was fixed and stained by treating for 10 min with a solution containing 1.8% Ponceau S, 26.8% trichloroacetic acid and 26.8% sulfosalicylic acid. The records above are densitometric scans of the fixed and stained cellulose acetate strips. Scan a was carried out at pH 7.0; scan b, at pH 7.6; scan c, at pH 8.5; and scan d, at pH 9.3 [From Yu and Masoro (25)].

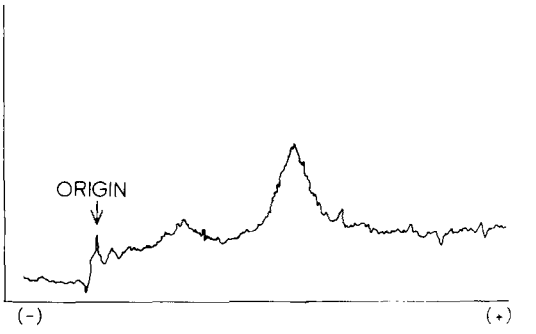

FIG. 14. Polyacrylamide-agarose gel electrophoretic analysis of fraction-2. The record above is a densitometric scan [From Yu and Masoro (25)].

about 17,000, a value reported in a preliminary communication (24). Shapiro and Maizel (27) recently reported further data on their method which suggested that estimation of the molecular weight of small proteins by 5% polyacrylamide gel electrophoresis may be in considerable error, a problem that can be circumvented by the use of higher percentage gels. Analysis of dissociated fraction-2 in higher percentage gels does indeed indicate that the original estimation of molecular weight is in error. With a 12% gel (Fig. 17), all protein entered the gel and migrated as a single band. On the basis of the electrophoretic migration distance of the dissociated fraction-2 protein relative to standard proteins its molecular weight is estimated to be approximately 6500 (Fig. 18). Electrophroetic analysis of dissociated fraction-2 in a 15% gel was totally consistent with the findings obtained with a 12% gel. Recently Weber and Osborn (28) and Dunker and Rueckert (29) have presented much evidence in support of the validity of the method of Shapiro et al. as means of determining the molecular weight of proteins.

Dissociation of the protein in fraction-2 and Sephadex G-150 column chromatography by the method of Pagé and Godin (30) also provided evidence for a single protein species. All of the protein eluted in a single sharp peak which, on the basis of its V_e/V_o ratio compared to that of standard proteins, indicated a molecular weight of 6500 (Fig. 19).

Identification of the N-terminal amino acid or acids in the protein of fraction-2 was done by preparing the DNP and dansyl derivatives. Only N-terminal glycine could be identified. Quantitative estimation by a densitometric technique (Fig. 20) indicated that glycine could account for all of the N-terminal amino acid present in the protein; of course such data are equivocal because of the losses which occur on

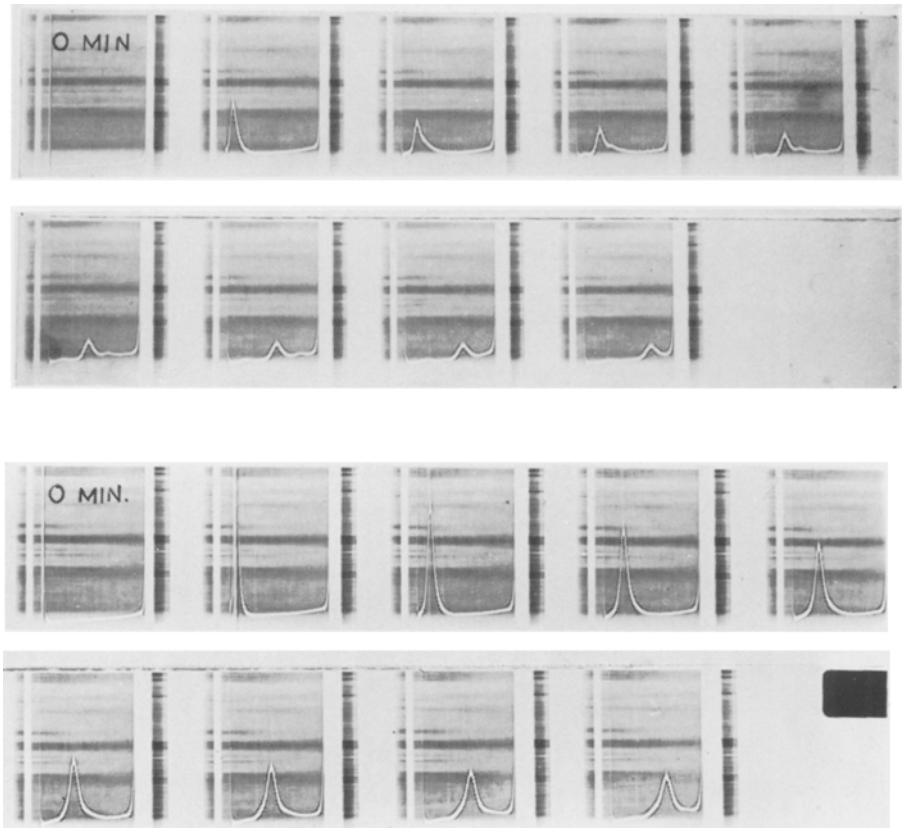

FIG. 15. Analytical ultracentrifugation of fraction-2. a, In presence of 2-mercaptoethanol; b, in absence of 2-mercaptoethanol. Pictures were taken at 16 min intervals after full speed was reached [From Masoro and Yu (24)], for experimental procedures see that reference.

preparation of DNP-derivatives (correction values obtained with standards probably only approximately apply to the protein in fraction-2) and because of the uncertainties in absolute value of a molecular weight based on electrophoretic and gel filtration analysis.

Both carboxypeptidase A and B were used for C-terminal analysis and the results indicated that alanine is a C-terminal residue (the data obrained with carboxypeptidase A are presented in Fig. 21). On the basis that alanine is the sole C-terminal amino acid present, the molecular weight of the protein subunits was calculated. The estimated value with carboxypeptidase A was 9880 and with carboxypeptidase B 10,300, results which agree rather well with the value of 6500 from the physical methods described above.

Peptide mapping of the protein in fraction-2 was carried out following tryptic digestion.

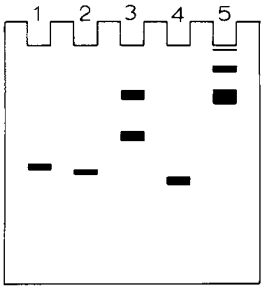

FIG. 16. Analysis of dissociated protein of fraction-2 by electrophoresis in 5% polyacrylamide gel containing dissociating agents. The slots are numbered 1 to 5 consecutively from left to right; slot 1 contains apo-ferratin; slot 2 fraction-2; slot 3, pepsin; slot 4, hemoglobin; slot 5, bovine serum albumin. Electrophoresis was carried out for 3 hr [From Masoro and Yu (24)]; for experimental procedures see that reference.

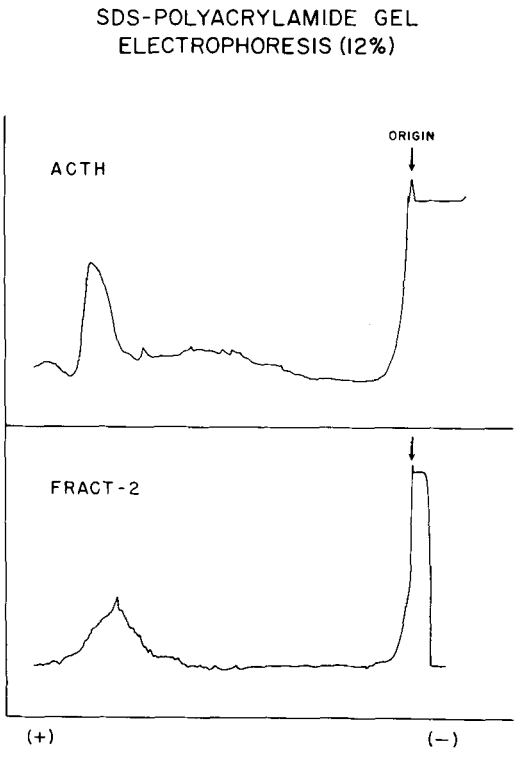

FIG. 17. Analysis of dissociated protein of fraction-2 by electrophoresis in a 12% polyacrylamide gel containing dissociating agents. Electrophoresis for 24 hr. Upper densitometric scan is for dissociated ACTH and lower scan for dissociated fraction-2 [From Yu and Masoro (25)]; for experimental procedures see that reference.

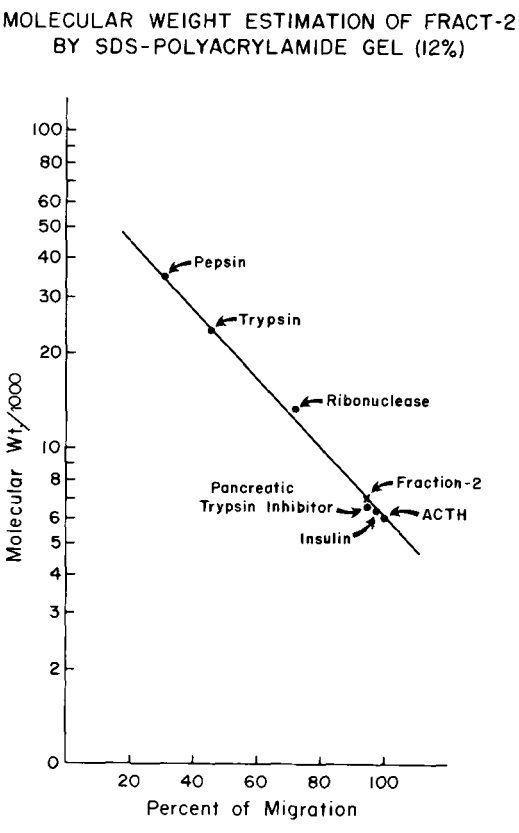

FIG. 18. Relationship between molecular weight of a dissociated protein and its migration distance in the 12% polyacrylamide gel containing dissociating agents. All proteins were treated and assayed as indicated in Figure 17 [From Yu and Masoro (25)].

Fifteen different peptides were detected (Fig. 22).

It seems clear that in the absence of dissociating agents but in the presence of some salt the protein in fraction-2 is composed of large water soluble aggregates of a polypeptide with a molecular weight in the range of 6500 to 10,500. That these aggregates are of very large size is established by their behavior on Sepharose and Sephadex columns, their sedimentation on analytical ultracentrifugation and their inability to enter a 4% polyacrylamide gel. The heterogeneity observed with undissociated fraction-2 probably results from variations in the extent of aggregation.

Upon dissociation, fraction-2 contains only small molecular weight components, e.g., all protein will enter a 15% polyacrylamide gel. The sole N-terminal amino acid present is glycine and probably the sole C-terminal amino acid is alanine. The molecular weight of these dissociated protein subunits is 6500, if estimated on the basis of the gel electrophoretic and the gel filtration methods, and it is approximately 10,000 if estimated on the basis of the C-terminal amino acid residue analysis.

On the basis of a single N-terminal amino acid species, a single migrating band of the dissociated protein on gel electrophoresis and a single sharp elution peak of the dissociated protein on gel filtration, it seems likely that the protein subunits of fraction-2 are either identical or very similar polypeptide species.

Some comment is in order, however, in regard to the peptide mapping data. Fifteen peptides were found, which is more than would be expected from a polypeptide of the composition reported in Table II if the molecular weight is 6500 but not if it is 10,000. Therefore the peptide mapping data are consistent either with the presence of only one polypeptide

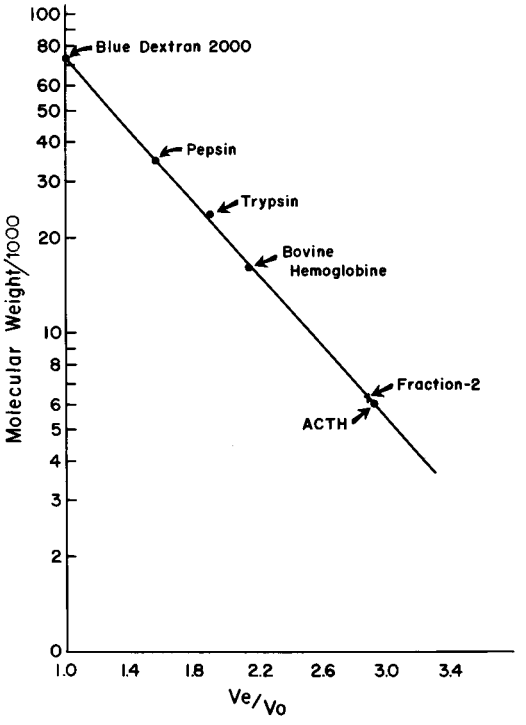

FIG. 19. Relationship between molecular weight of a dissociated protein and its elution behavior on a SDS-containing Sephadex G-150 column. The protein of fraction-2 and the standard proteins at a concentration of 6 mg/ml were preincubated with 0.075 M SDS containing 1.5 x 10^{-4} M *p*-hydroxymercuribenzoate at pH 8.0 at 37 C for 24 hr prior to Sephadex gel filtration. Sephadex G-150 was equilibrated in a 0.05 M SDS solution containing 1.5 x 10^{-4} M *p*-hydroxymercuribenzoate at pH 8.0. The eluent had the same composition as the equilibrating solution. Chromatography was carried out at room temperature at an elution rate of 3-4 ml/cm^2/hr [From Yu and Masoro (25)].

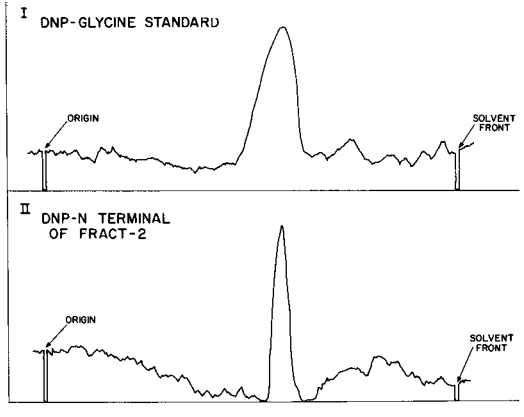

FIG. 20. Thin layer analysis of DNP derivatives prepared from fraction-2. The above is a scan of ether extractable acidified-DNP derivatives. The nonether extractable fraction was similarly studied and no evidence for the presence of an N-terminal derivative was found [From Yu and Masoro (25)].

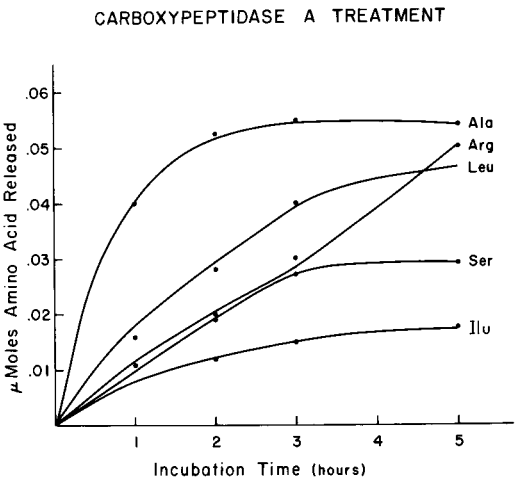

FIG. 21. Rates of release of amino acids by carboxypeptidase A treatment of fraction-2 [From Yu and Masoro (25)]; for experimental procedures see that reference.

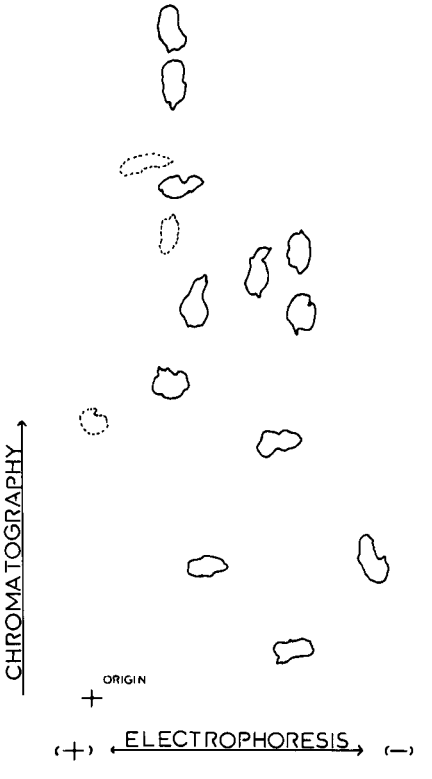

FIG. 22. Peptide mapping of fraction-2. The peptides are detected by spraying with 0.2% ninhydrin reagent in *n*-butanol saturated with water. Color is developed by heating. Spots outlines by dashed line exhibited only faint color [From Yu and Masoro (25)]; for experimental procedures see that reference.

species with a molecular weight of 10,000 or at most of two polypeptide species both with a molecular weight of 6500 and with a similar but not identical primary structure.

Martonosi (31) recently reported that he was not able to confirm our finding that the dissociated protein from sarcotubular membranes migrates as a single band on polyacrylamide gel electrophoresis. It is difficult to give a reason for this discrepancy but it may be related to the presence of varying amounts of lipid combined with the protein since he provides no information on the preparation of the soluble membrane protein nor on its chemical composition. In our experience only complete dissociation of lipid-poor sarcotubular protein yields a preparation which appears homogeneous on gel electrophoresis.

Since fraction-2 contains approximately 90% of the protein of sarcotubular membranes, it appears that we have isolated the protein structure responsible for the molecular architecture of thesy membranes. Moreover, if Mommaerts is correct in concluding that most of the protein in the sarcotubular membrane is involved in Ca^{++} transport, then this protein is the one responsible for Ca^{++} transport. It is tempting to contemplate utilizing this protein for a stepwise reconstitution of both the membrane structure and its Ca^{++} transport activity. However, naturally occurring membranes have a lipoprotein structure (22,31) and, although it is important to isolate the proteins of membranes in order to chemically identify and characterize them, it remains to be established that a protein so obviously denatured can be used for reconstitution of the membrane system. Success will depend on the extent to which such protein can be "renatured" by, e.g., the introduction of lipid to reform a lipoprotein structure.

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Serum Lipid Transport Systems: Recent Advances[1]

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ABSTRACT

Lipids circulating in the plasma are transported in water soluble form as lipid-protein complexes. Lipoproteins can be classified according to size, density, electrophoretic mobility and protein composition. The ability of low density lipoproteins and very low density lipoproteins (VLDL) to form complexes with different polyanions has been also used as a method for separation and study of serum lipoproteins. Even within classes of lipoproteins closely related otherwise, the amount of different lipids and their ratios to each other and to protein are variable. Two enzymatic systems seem to be at least partly responsible for the different lipid compositions of serum lipoproteins: lipoprotein lipase and lecithin-cholesterol acyltransferase (LCAT). LCAT, which seems to be associated with α-lipoproteins, is responsible for the formation of the bulk of cholesteryl-esters in human serum. Changes in activity of this enzyme may explain the observed changes with age and disease of serum cholesteryl-ester fatty acids (CEFA). Differences in CEFA pattern are found between newborn and adult animals, including man. The activity of serum LCAT was observed to increase with age in animals and to decrease markedly in patients with liver cirrhosis. These patients show abnormal serum CEFA patterns and abnormally low proportions of pre-β- (VLDL) and α- (high density) lipoproteins.

INTRODUCTION

At the International Symposium on Lipid Transport celebrated in Nashville in 1963 it was predicted that the "finding of specific proteins as lipid acceptors was very exciting and that further study of these apoproteins will bring about a better understanding of how lipids are transported in the blood" (1). This prediction proved to be true although a great deal still remains to be learned.

In this paper no attempt will be made to present a comprehensive review of all the advances in the field of lipoprotein and lipid transport that have occurred since the 1963 meeting. We will discuss selected aspects of recent advances in methodology which have led to a better understanding of the structure and function of lipoproteins. We will discuss new advances in our knowledge of enzymes which are known to participate in lipoprotein metabolism and function and, finally we will discuss the transport of a specific serum lipid fraction which is suspected of having an important physiological role, the cholesteryl-esters.

ADVANCES IN LIPID METHOLOGY

In the past few years important advances have been made in this area. A summary of the techniques currently used in the study of lipoproteins follows.

1. differential salt precipitation
   Macheboeuf (1929), Adair and Adair (1944)

2. Cohn cold ethanol fractionation
   Gurd, Oncley, Edsel and Cohn (1949), Cohn (1950)

3. analytical ultracentrifugal flotation
   Pederson (1945), Gofman et al. (1951)

4. preparative ultracentrifugation, followed by chemical analysis
   Lindgren et al. (1951), Hillyard et al. (1955),
   Havel et al. (1955), Bragdon et al. (1956)

5. complexing with polyanions
   Walton (1952), Oncley and Mannick (1954),
   Burstein and Samaille (1955), Bernfeld et al. (1957)

6. electrophoresis
   a. moving boundary electrophoresis
      Blix, Tiselius and Swensson (1941)
   b. zone electrophoresis
      i. starch block, Kunkel and Slater (1952)
      ii. paper, Swahn (1953), Durrum et al. (1952), Straus and Wurm (1958),

[1]One of five papers to be published from the Symposium "Lipid Transport" presented at the AOCS Meeting, New Orleans, April 1970.

Lees and Hatch (1963)
iii. starch gel, Smithies (1955)
iv. cellulose acetate
v. acrylamide, Narayan et al. (1965)
vi. agar and agarose gel, Graber and Williams (1955) Ressler et al. (1961), Nobel (1968)
c. immunoelectrophoresis, Graber and Burtin (1964)

7. immunodiffusion
Ouchterlony (1953), Mancini (1965)

8. membrane filtration
Stone and Thorp (1966)

9. others
a. nuclear magnetic resonance, Chapman (1968)
b. refractometry, Lindgren et al. (1960)
c. electron microscopy, Hayes and Hewitt (1957)
d. x-ray
e. spectroscopy
i. infrared, Scanu and Granda (1968)
ii. circular dichroic, Scanu and Hirz (1968)

The two techniques most widely used have been ultracentrifugation, either ultracentrifugal flotation or ultracentrifugation followed by chemical and physiochemical analysis, and different types of electrophoresis. In general, good correlation has been found between electrophoretic and ultracentrifugally isolated lipoproteins. These correlations have been extremely useful in establishing bridges of communication between clinicians and biochemists and have permitted a better understanding of the physiological role of lipoproteins.

On the basis of density, flotation, electrophoretic behavior and amino acid residues, four main groups of lipoproteins have been characterized (Table I): 1) chylomicrons, 2) very low density lipoproteins (VLDL) or pre-β-lipoprotein by electrophoresis, 3) low density lipoproteins (LDL) or β-lipoproteins by electrophoresis and 4) high density lipoproteins (HDL) or α-lipoproteins by electrophoresis. The presence of four major residues, aspartic acid, glutamic acid, serine and threonine as N-terminal amino acids, in these lipoproteins suggests that there must be at least four different apoproteins forming part of the lipoproteins. We will discuss this point later.

There are techniques used in the study of lipoproteins based on the ability of LDL and VLDL to form complexes with different polyanions (2,3). We have studied the formation of these complexes using different mucopolysaccharides (MPS), cations, etc. (4) and found that at physiological pH, maximum complex formation takes place at concentration of Ca^{++} of 20 mg/ml of serum and at concentrations of heparin below 2 mg/ml serum (5). At higher concentration of heparin, other serum proteins in addition to β-lipoproteins are coprecipitated. The presence of N-sulfated groups in the hexosamine residue of MPS increased their complexing ability with the β-lipoproteins. We have concluded that in the formation of these complexes between LDL, VLDL and MPS, Ca^{++} seems to interact first with phosphate groups of phospholipids of lipoproteins, thereby providing catonic sites to the reacting group of MPS. However, other types of interaction such as electrostatic forces, hydrogen bonds or hydrophobic bonds cannot be excluded as participating in the formation of the com-

TABLE I

Major Classes of Human Serum Lipoproteins

Lipoprotein class	Flotation Sf.	Density range (g/ml)	Paper electrophoresis	N terminal aminoacids Major	Minor
Chylomicrons	>400	<0.95	Origin	Ser Thr	Glu Asp
VLDL[a]	20-400	0.95 -1.006	pre-β	Ser Thr	Glu Asp
LDL	0-20	1.006-1.063	β	Glu	Ser Thr
HDL	---	1.063-1.21	α	Asp	

[a]Abbreviations: VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; Ser, serine; Thr, threonine; Glu, glutamic acid; and Asp, aspartic acid.

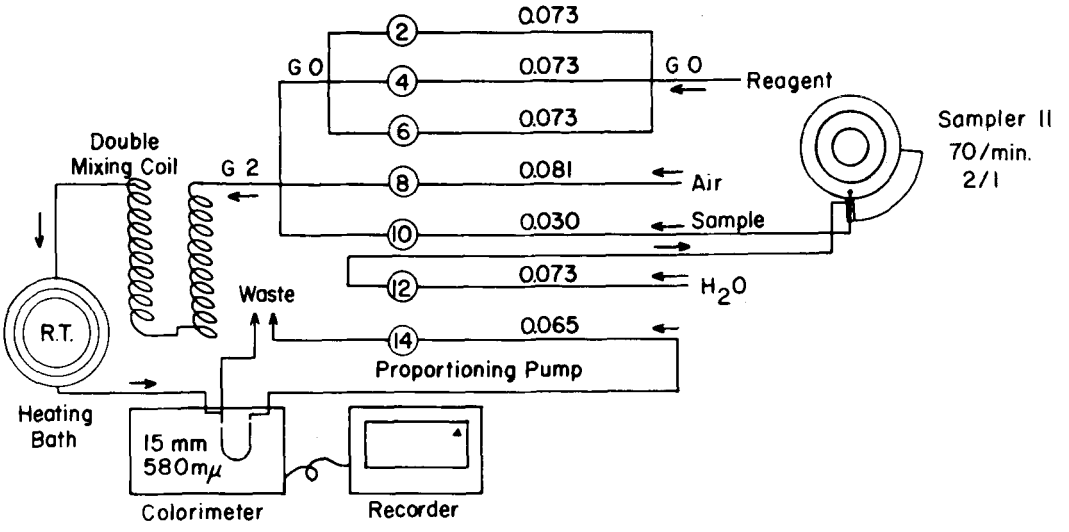

FIG. 1. Flow diagram of automated methods. Heparin Reagent consists of 0.25% heparin, 0.5 M $CaCl_2$ and H_2O in the following proportions: Heparin, 1; $CaCl_2$, 2.5; and H_2O, 15.5 volumes. Reagent blank is prepared as the heparin reagent with the omission of heparin.

plexes. Recently, Day and Levy (6) have reported that the interaction of LDL with polyanions (amylopectin, heparin) is ionic in nature and that free amino groups are essential for the interaction. It is possible that the trapping of β-lipoproteins in the arterial wall by acid MPS is basic to the pathogenesis of atherosclerosis.

Whatever the mechanism of interaction is, the ability of complex formation between β-lipoproteins and polyanions has been used as a means of separation and quantitation of LDL plus VLDL; we have found a very good correlation between the amount of cholesterol in the precipitated β-plus pre-β-lipoproteins and the turbidity value obtained after 15 min incubation of serum, heparin, $CaCl_2$ and water in appropriate amounts (7). Furthermore, we have been able to automate this procedure (Fig. 1) and in this way we can estimate rapidly, efficiently and accurately (Fig. 2) the amount of cholesterol present in the β-plus pre-β-lipoproteins of serum at a rate of 35 or 70 determinations per hr. The details of this method will be published elsewhere (8).

Techniques have also been devised to study lipoproteins based on their immunological properties. These properties are mostly associated with the LDL and VLDL (9); the important lipid component of these lipoproteins is haptene (10). HDL and LDL seem to have antigenic specificities which are not shared. On the other hand, VLDL, unmodified and unchanged, is immunochemically identical with LDL. Heterogeneity in the form of genetic polymorphism occurs in plasma lipoproteins in the same manner as it occurs in other plasma proteins. Polymorphic forms of LDL were first described by Blumberg (11) and designated Ag. Since then, a number of Ag have been described in the context of the immunological reactions of lipoproteins, whose specie specific is not absolute and whose group specific is associated with lipoproteins, is heteroimmune in the Lp system—Lp(a) positive and Lp(a) negative and is isoimmune in the Ag system—As(A_1), Ag(x), Ag(z), Ag(b), Ag(c), Ag(t), Ag(y) and Au. A second LDL system, Lp, has been described by Berg (12), who concluded that these two antigenic systems are independent and that the two antigens appeared to be on different parts of the same LDL molecule. More recently, it has been reported (13) that the Lp antigenic determinants are peculiar to a distinct class of lipoprotein, "the sinking prebeta" (SPB) lipopro-

TABLE II

APO-Lipoproteins
(COOH-Terminal Residues)

APO VLDL[a]	APO LDL	APO HDL
R-Ser	R-Ser	R-Glu
R-Ala		R-Thr
R-Val		
R-Thr[b]	R-Ala[b]	R-Ala[b]
R-Glu[b]	R-Val	R-Val[b]

[a]Abbreviations: See Table I. Also, Ala, alanine; Val, valine.

[b]Present only in small quantities.

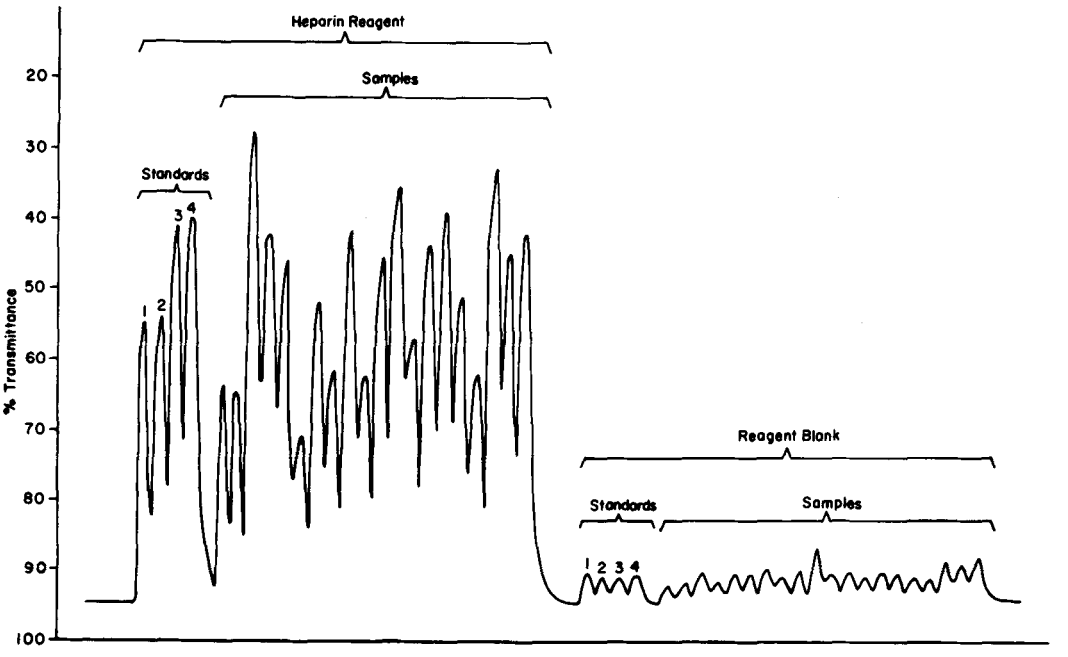

FIG. 2. Tracing obtained with standards and unknowns.

tein and that SPB is the Lp antigen. Immunochemical techniques have been very useful in the study of lipoprotein deficiency states (14), quantitation of lipoproteins (15), demonstration of the purity of lipoprotein preparations (7) and in the study of apoproteins (16).

Thus, using immunochemical techniques it has been found (17,18) that human lipoproteins contain at least five distinct peptides (Table II); three of these comprise the major protein constituents of the LDL and HDL (apo LDL-serine, apo HDL-glutamine and apo HDL-threonine). In the VLDL three peptides have been reported recently (19) which are different from the others; they are apo VLDL-valine, apo VLDL-alanine and apo VLDL-glutamic acid. These peptides form part of the different apoproteins. Work by Alaupovic et al. (20) and Shore and Shore (21) indicated that each of the major lipoprotein classes isolated on basis of density is a mixture of lipoproteins differing in the nature of the protein moiety as well as in lipid content. For this reason a classification of lipoproteins based on the type of apoprotein present has been proposed by Alaupovic (22) in which the usually reported density ranges of the major lipoprotein classes, VLDL, LDL, HDL and VHDL, are presented at the same time that the heterogeneity of these fractions can be observed in respect to their protein moieties.

Although the exact configuration of the lipoprotein is not known, on the basis of information available from the literature and their own work, Day and Levy (23) have proposed for HDL_2, HDL_3 and LDL a system of small repeating units called lipotides. These units consist of two helical peptide chains enclosing a lipid core, each helix being attached to the other by a nonhelical peptide connector. Pollard et al. (24) have suggested, as a model for LDL, a dodecahedron with one protein subunit at each of the twenty vertices; the phospholipids are probably located on the faces of the dodecahedron and neutral lipids in the interior.

The structures of VLDL and chylomicrons seems to be micellar in nature, with phospholipid-protein complexes bound at the surface to stabilize the micelle. The proportion of surface covered by protein in the VLDL seems to be approximately 20% (25). Another model of lipoprotein structure has been more recently suggested by Gotto (26). In this, as in the previous models, the polar side of the surface coat would be exposed to the surrounding aqueous medium while the apolar side would interact with the hydrophobic core of triglycerides and cholesteryl-esters. All these models are compatible with the idea of direct lipid interchange among different lipoproteins (27,28).

Other techniques, such as nuclear magnetic resonance and spectroscopy, have given some insight about the nature of the bond between

lipids and proteins in the lipoproteins, and suggest that the interaction between lipids and protein is predominantly polar in nature rather than hydrophobic (29); however, more work is necessary for a better understanding of the nature of the lipid protein interaction in lipoproteins.

ENZYMES INVOLVED IN LIPOPROTEIN METABOLISM

On the basis of their lipid constituents, we can consider two types of lipoproteins (31), triglyceride-rich lipoproteins (chylomicrons and VLDL) and cholesteryl-ester and phospholipid-rich lipoproteins (LDL and HDL). The first group is probably involved in some form of energy metabolism involving triglyceride transport, and the second, in the metabolism and transport of cholesteryl-esters. However, from the functional point of view, there seems to be a great deal of interrelationship between the lipoproteins of these two groups. We mentioned earlier the chemical and immunochemical similarities between VLDL and LDL. There is also evidence that lipids can be interchanged between lipoproteins of different classes (27,28) and metabolic interrelationships between VLDL, LDL and HDL have been reported (30,31).

The removal of serum triglycerides (exogenous or endogenous) from the blood stream appears to require hydrolysis which is catalyzed by the enzyme lipoprotein lipase (E.C. 3.1.1.3) located near the surface of the capillary endothelium. These triglycerides are hydrolyzed only in the presence of lipoproteins which appear to be necessary for formation of enzyme-substrate complex. The activity of this enzyme, lipoprotein lipase, seems to be associated with the C-terminal peptide R-Glutamic acid of the HDL and VLDL (32-34).

LDL and HDL are generated in the process of triglyceride removal and as end products of chylomicrons and VLDL breakdown (35,36). The LDL which are formed are comparable to the normally occurring LDL, whereas the HDL contain more phospholipids and less of the other lipid components found in normal HDL (37); these HDL will be converted to normal HDL by the action of another enzyme, lecithin-cholesterol-acyl-transferase (LCAT) (E.C. 2.3.1), which catalyzes the transesterification reaction between lecithin and cholesterol in plasma.

Havel has proposed the following model of lipoprotein metabolism and function (38). Upon entrance into the circulation, chylomicrons and VLDL acquire apo A from HDL or

TABLE III

Serum CEFA Patterns and LCAT Activity at the Time of Delivery

	27 mothers, mg/100 ml	27 babies, mg/100 ml
Triglycerides	185	47
Total cholesterol	235	71
Free	50	20
Esterified	186	52
S[a]	27	10
O	55	22
L	86	10
A	18	10
O/L	0.6	2.2
LCAT activity	3.9[b]	2.6[b]

[a]Abbreviations: S, cholesteryl-esters of saturated fatty acids C:16, C:18; O, cholesteryl-oleate; L, cholesteryl linoleate; A, cholesteryl-arachydonate; and O/L, cholesteryl-oleate:cholesteryl-linoleate.

VLDL. Circulating apo A, once bound to VLDL, may serve to facilitate the action of lipoprotein lipase, LCAT, or both. Lipoprotein lipase will remove triglycerides from chylomicrons and VLDL, thus causing a reduction in the volume of these lipoproteins. But, at the same time, LCAT will remove lecithin and cholesterol, enabling lipoproteins in this manner to retain their shape and properties at the time when triglycerides are removed. One product of the LCAT, lysolecithin, is transported to VLDL, or to albumin, and the other, the esterified cholesterol, might enter the interior of the lipoprotein or is transferred to LDL or HDL lipoproteins.

The LCAT enzyme seems to be mainly responsible for the concentration of cholesteryl-esters in the plasma (39). Although lecithin is the main source of fatty acids for the transesterification brought about by LCAT enzyme (40), there is evidence indicating that triglycerides may contribute as fatty acid donor for the reaction (41). The enzyme has specificity for the fatty acid in position number two of the lecithin (40), and this explains the preponderance of unsaturated fatty acids in the serum cholesteryl-esters (42,43). This enzyme is closely associated with HDL (44), but can be separated from them (45); four times as much HDL-cholesterol is esterified as LDL-cholesterol (46). An esterifying enzyme of similar specificity as the one in the plasma has been detected in the soluble fraction of liver homogenates (47) and in other tissues (48), but the concentration in plasma is much greater than in the other tissues. It is possible, therefore, that the

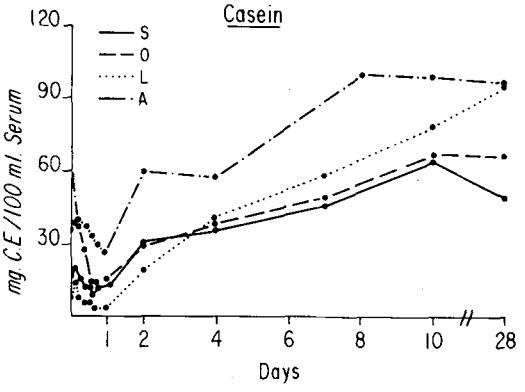

FIG. 3. Changes in serum cholesteryl-esters of young rats during the first month of life whose mothers received a diet containing 18% casein.

enzyme may become active after being released from the liver to the circulation. Marsh and Kashub (49) have presented evidence suggesting that the liver is the source of the plasma LCAT. Sulfhydryl groups are important in the activity of the enzyme (50), and its activity is known to change with age (51), sex (51), diet (52) and substrate concentration (53). A genetic disease due to absence of this enzyme has been described (54).

It is obvious that this enzyme has a very important role in lipoprotein metabolism, and Glomset (50) has proposed that LCAT plays a role in the transport of cholesterol from membranes, including those of red blood cells (37), to the liver.

CHOLESTERYL-ESTERS

A summary of the blood lipids and their concentrations in humans follows: (a) Total lipids, 400-1000 mg/100 ml. (b) Cholesterol, 150-230 mg/100 ml (esters, 125-172 mg/100 ml; free, 25-58 mg/100 ml). (c) Triglycerides, 40-160 mg/100 ml. (d) Phospholipids, 120-250 mg/100 ml (lysolecithin, 7.9 ±2.6% of total; sphingomyelin, 17.0 ±5.0% of total; lecithin, 66.0 ±10.5% of total; phosphatidyl-serine-inositol, 2.8 ±3.0% of total; phosphatidyl-ethanol-amine, 4.4 ±2.1% of total). (e) Free fatty acids, 0.3-0.7 mEq/L. (f) Carotenoids, 0.07-0.2 mg/100 ml. (g) Vitamin A, 0.02-0.07 mg/100 ml. (h) Vitamin E, 1.05 ±0.26 mg/100 ml. (i) Cerebrosides (expressed as hexose), 3.5-5.7 mg/100 ml. (j) Glycolipids, 1.8 mg/100 ml. (k) Squalene, 30-35 μg/100 ml. As can be seen, cholesterol is one of the largest lipid fractions to appear in the blood, and two thirds of this cholesterol is esterified with different fatty acids. The relative proportion of free to esterified cholesterol in the plasma is generally considered to be relatively constant, and there are reasons to believe that the study of each of the individual cholesteryl-esters will lead to a better understanding of the physiological role of this serum lipid fraction.

In 1965 we published evidence (55) confirming the existence of different types of cholesteryl-esters previously described by other authors (56): an "adult" type in which cholesteryl-linoleate predominates over cholesteryl-oleate and an "infant" type in which the reverse is observed, i.e., a predominance of cholesteryl-oleate over cholesteryl-linoleate. We found a cholesteryl-oleate: cholesteryl-linoleate (O/L) ratio of 0.6 for pregnant rats at the time of delivery and an O/L ratio of 1.5 for their babies. Similar findings in humans have been reported in the literature by Zollner et al. (57), Muldrey (58), and by us (59). In two groups of 27 mothers and of 27 babies, we have found (Table III) that the newborn babies have, in addition to the differences in the O/L values, lower LCAT activity than their mothers. The changes from the "infant" to the "adult" type of cholesteryl-ester fatty acids (CEFA) in rats were found to occur during the first week of life (Fig. 3) and in humans during the first three months of life (A. Lopez- S., unpublished data). We found (60) that the changes observed in serum CEFA with age were accompanied by changes in the enzymes from liver homogenates that hydrolyze and esterify cholesterol and by

TABLE IV

Per Cent Distribution of Incorporated Free Cholesterol 4-C^{14}

Cholesteryl esters[a]	1-Day-olds	3-Day-olds	6-Day-olds	Adults
S[b]	9.7	21.4	23.3	21.2
O	58.0	21.8	22.6	23.3
L	22.6	17.6	24.9	25.6
A	9.7	39.0	29.2	29.9

[a]Incubation mixture: 0.5 ml of serum, 0.85 μg of 4-C^{14}-cholesterol, 25 mg of celite.
[b]Abbreviations in Table III.

TABLE V

Cholesteryl-Oleate:Cholesteryl-Linoleate Ratios

	Iowa[a]	Honduras[b]
Mean O/L value	0.60%	0.67%
Range of .80 - .90	5.5%	7.2%
Range of .90 - 1.0	2.4%	2.5%
>1.0	4.4%	9.1%
Above 0.8 (total)	11.3%	18.8%

[a]Number of subjects, 1628.
[b]Number of subjects, 1030.

changes in serum LCAT activity. It was found also that as an effect of LCAT activity (Table IV) more C^{14}-cholesterol is incorporated into cholesteryl-oleate than into cholesteryl-linoleate in the newborn animals, whereas the reverse was observed in adult rats.

In humans, we have described (43) the changes which occur in the CEFA with advancing age in 1,628 individuals from the state of Iowa, ranging from 12 to 70 years of age. It was found that the slope for total cholesterol is larger than the annual change of the total esterified fraction, which implied that the nonesterified fraction of cholesterol increased with age. This tendency toward an increase in free cholesterol with age may be explained on the basis of decreased activity with age in the LCAT enzyme (51,60) which has been

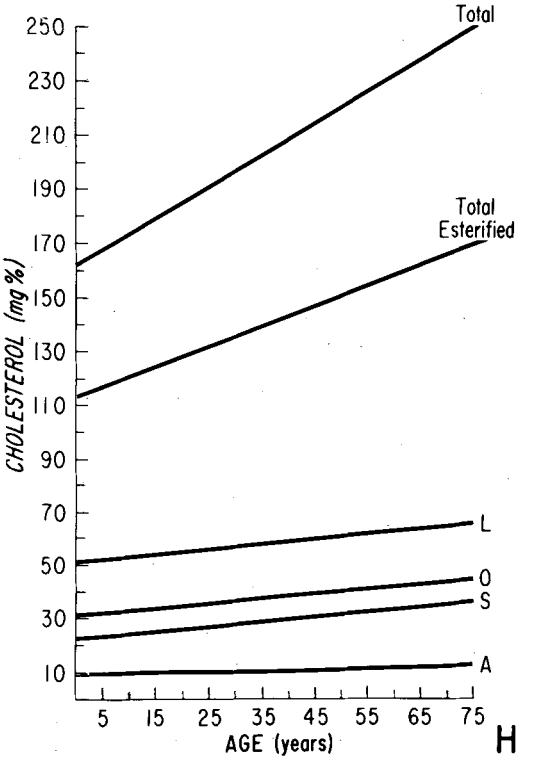

FIG. 4. Regression lines of the changes with age of plasma total cholesterol and cholesteryl-esters in 1,030 individuals from the Republic on Honduras.

TABLE VI

Serum CEFA, LCAT Activity and Lipoprotein Concentrations in Patients With Liver Cirrhosis as Compared With Normals

	Liver cirrhosis, mg/100 ml		Normals, mg/100 ml	
Total cholesterol	156	(6)[a]	191	(69)[a]
Free cholesterol	63	(6)	47	(69)
CEFA:				
S[b]	10	(6)	21	(6)
O	38	(6)	47	(6)
L	24	(6)	74	(6)
A	7	(6)	5	(6)
O/L Ratio	1.58		0.63	
Lipoproteins:				
β	241	(7)	232	(69)
Pre-β	1.3	(7)	81	(69)
α	181	(7)	286	(69)
LCAT	0.7[c]	(7)	6[c]	(6)

[a]Number of subjects in parentheses.
[b]Abbreviations in Table III.
[c]μM/24 hr/ml.

described by different investigators. This decrease in LCAT activity also brings about changes in lipoprotein distribution with an increased proportion of VLDL with advancing age. Since the ratio of esterified to free cholesterol in β-lipoproteins falls as the density of the lipoprotein decreases, variations in lipoprotein distribution is probably the major reason for changes in the proportion of plasma free cholesterol (61). We observed similar trends with age of serum CEFA in a group of 1,030 sera from the Republic of Honduras, Central America (Fig. 4). However, the presence of O/L ratio of the "infant" type mentioned before was larger in the Honduran sample than in the sample from the USA (Table V) (62). This could be explained on the basis of reported larger values of VLDL in Central American populations (63,64). As mentioned before, there seems to be a selective incorporation of the polyunsaturated cholesteryl-ester into the high density lipoproteins due to the activity of the LCAT enzyme, whereas the esterifying enzyme in the soluble fraction of the liver will favor the incorporation of saturated and monounsaturated cholesteryl-esters in the lower density lipoproteins (65) whose proportion is apparently increased in the Central American population.

Abnormalities in serum CEFA patterns, with O/L ratios above 1.0, have been also described in malnourished patients (66) and in patients with liver disease (67). We have confirmed these findings in patients with liver cirrhosis (Table VI) and found that these patients also have reduced activity of LCAT enzyme and reduced concentration of α-lipoprotein. It remains to be established if the reduced concentration of cholesteryl-linoleate, as compared with cholesteryl-oleate in these patients, is due to a decreased production of LCAT enzyme by the liver or to decreased substrate (α-lipoproteins are decreased), or if it is due to decreased albumin (normal acceptor of lysolecithin, a products of the transferase reaction), or to decreased synthesis of lecithin, or even to a release by the liver of hydrolase for cholesterol hydrolysis. Our present studies are aimed in this direction. Recent evidence (68) suggests that the low LCAT activity of patients with liver disease is most probably due to impaired synthesis or release of the enzyme by the damaged liver.

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Lipid Alterations and Their Reversion in the Central Nervous System of Growing Rats Deficient in Essential Fatty Acids[1]

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ABSTRACT

Essential fatty acid (EFA) deficiency modifies several biological parameters, i.e., growth, metabolic rate and water balance and induces functional changes in liver, kidney and lung tissues. The brain fatty acid changes reported in the literature are generally smaller than those observed in other tissues. However, EFA deficiency initiated in rats prior to birth and continued for a prolonged period of time results in decreased brain weight, brain lipid and phospholipid content and in considerable changes in polyunsaturated fatty acid distribution, especially in the ethanolamine phosphoglyceride fraction. The unsaturation level of this phospholipid is maintained at a constant level in spite of the fatty acid distribution changes. Brain lipid analyses, carried out at various time intervals in EFA deficient animals, indicate that brain weights are already reduced in 10-day-old deficient rats, that brain phospholipids decrease, especially in the males after three months, and that fatty acid distribution changes begin rather early. The latter consist of a decrease of tetraenes and increase of trienes and of the triene-tetraene ratios as early as at 10 days, a decrease of hexaenes after six months, and after six months, an increase of pentaenes, which are elevated at birth but usually disappear after three months of age. Similar changes are observed in myelin. The induced changes are not completely reversed upon return to normal diet.

INTRODUCTION

The essential role of dietary polyunsaturated fatty acids (PUFA), which belong to the linoleate and linolenate families, in balanced body growth and proper function of several tissues and organs has been recognized many years ago and extensively investigated during the last two decades.

Recent reviews of the literature concerning essential fatty acid (EFA) deficiency have been presented by Holman (1) and by Alfin-Slater and Aftergood (2), while the metabolism of PUFA has been discussed by Mead (3). Investigations on the effects of EFA deficiency at the biochemical level have elucidated changes in the pattern and metabolism of PUFA in tissue lipids. The major modifications detected in the tissues of EFA deficient animals consist of a decrease of PUFA of the linoleate (18:2 w6) and linolenate (18:3 w3) acid families and an increase of trienes, especially 20:3, derived from oleic acid (18:1 w9).

The fatty acid changes observed in brain during EFA deficiency (4-7) are generally less pronounced than those occurring in other tissues (4,5,8-11,14). This has been attributed to the lower turnover of brain fatty acids which are part of the complex lipids located in cell and subcellular membranes of the perennial nervous cells. It should be taken into consideration that most of the experiments reported in the literature have been carried out on weanling animals, i.e., the dietary EFA deficiency was induced after the formation and deposition of most of the brain lipids.

In contrast to the limited effect on the adult brain, nutritional deficiencies have a considerable influence upon growth and maturation of the central nervous system in experimental animals (12) and man (13) when induced during the period of brain development. The importance of dietary lipids for the growing brain is shown by the influence of the maternal diets upon the brain fatty acid composition in newborn rats (14). Furthermore, Steinberg et al. (15) have shown that, when female rats are raised to sexual maturity on an EFA deficient diet and then bred, the newborn's body and brain weights are reduced and the brain fatty acid composition is changed.

On the basis of these observations we proceeded to follow the effect on brain lipid composition and fatty acid distribution of EFA

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TABLE I

Body and Brain Weights in Control and EFA Deficient Male Rats

Days	Control		Deficient	
	Body weight, g	Brain weight, mg	Body weight, g	Brain weight, mg
10	21 ± 0.4 (11)[a]	953 ± 14	18 ± 0.4 (9)	858[b] ± 7
30	46 ± 1.1 (4)	1387 ± 8	45 ± 1.8 (4)	1346[c] ± 34
60	177 ± 10 (6)	1702 ± 37	108[b] ± 7.3 (5)	1655[c] ± 24
90	375 ± 9 (4)	1910 ± 54	128[b] ± 2 (3)	1516[b] ± 48
365	586 ± 40 (4)	2230 ± 90	417[b] ± 32 (6)	2000[b] ± 30

[a]The number of animals in parentheses.
[b]Significantly lower than control values $P<0.01$.
[c]Significantly lower than control values $P<0.05$.

deficiency initiated when brain development is most active. This was accomplished by feeding the deficient diet to rats during pregnancy and lactation and then to the offspring after weaning. The investigation has been carried out in order to study changes in brain lipid and phospholipid concentrations after different periods of EFA deficiency, focused on the fatty acid modifications of ethanolamine phosphoglyceride (EPG), a compound contained in high concentration in brain and particularly rich in polyunsaturated essential fatty acids.

The ability of tissues to recover from EFA deficiency has been studied by Walker (16,17). Fatty acid changes of brain total lipids are reversed more slowly than those occurring in other tissues in rats returned for 81 days to a control diet after a 25-week period of EFA deficiency (17). On the basis of these data, in our experiments we have also been interested in obtaining more detailed information concerning the degree of reversibility of fatty acid changes of specific brain phospholipids at different stages of EFA deficiency in rats maintained on deficient diets from the initial period of brain development.

MATERIALS AND METHODS

Animals and Diets

Pregnant rats of the Sprague-Dawley strain were fed ad lib. with either an essential fatty acid free diet (18) or a control diet with a 2% corn oil added, starting approximately one week before delivery. The control diet contained linoleate in the proportion of 0.6% on a weight basis (or 1.5% of the calories) and linolenate in the proportion of 0.02% of weight (or 0.05% of the calories) while only trace amounts of these acids were detected in the deficient diet. The treatment was continued during lactation and then these diets were fed to the offspring. Groups of control and deficient rats, subdivided into males and females, were killed at 3, 10, 30, 60, 90, 180 and 365 days of age.

An additional four groups of pregnant rats were fed on the EFA deficient diet starting five days before delivery. Two of these groups returned to the control diet at 10 days and this dietary treatment was continued for a further period of 20 and 50 days respectively. The weanlings were sacrificed at 30 and 60 days of age. The third and fourth groups of newborn EFA deficient rats returned to the control diet at 30 and 90 days of age respectively; the former were killed at 60 days and the latter at 180 days. Body and brain weights were measured. Brains of all groups were pooled, with the exception of the 60-day-old control and deficient groups, which were examined individually in order to evaluate the individual variability of the analytical data within these groups.

Lipid Analysis

Total lipids were extracted (19) and aliquots

TABLE II

Brain Phospholipid Concentrations (mg/g fresh weight) in 10-, 30-, 90-, 180-, and 365-day Old Control and Deficient Rats[a]

Days	Control A	Deficient B
10	27.2 (26.8-27.1-27.3)	27.6 (26.1-27.3-27.4)
30	48.2 (47.8-48.1-48.7)	51.6 (51.1-51.7-52.0)
90	45.5 (45.0-45.9-45.9)	40.1 (39.7-40.2-40.4)
180	51.9 (50.3-52.1-53.3)	45.8 (44.7-45.2-47.3)
365	46.0 (46.5-46.2-45.3)	40.7 (41.1-40.8-40.2)

[a]The values are the mean of three determinations made on the lipid extracts from the pooled brains. Values of individual determinations are also given.

of the crude lipid extracts were taken from the 10-, 30-, 90- and 180-day-old control and deficient groups, dried under vacuum, reextracted and the total lipid phosphorus was determined. An aliquot of the lipid extracts from each of the 180-day and 1-year-old rat groups was chromatographed on Sephadex G-25 (20) in order to determine the brain total lipid content.

Chromatography of Lipids

The total lipid extracts from all animal groups were chromatographed on one-dimensional thin layer chromatograms in order to purify ethanolamine phosphoglyceride (EPG). The solvent system employed was chloroform-methanol-acetic acid-water 80:20:5:2 and the adsorbent was a mixture of silicic acid and magnesium silicate (19). The EPG band was located after spraying the chromatograms with water, then it was dried under nitrogen and scraped off for fatty acid methyl ester preparation.

Fatty Acid Analysis

Fatty acid methyl esters deriving from both the mono- and diacyl forms of EPG were prepared and freed from aldehydes (21). Fatty acid analysis was carried out by gas liquid chromatography using a 2m x 3mm (i.d.) glass column packed with 5% polydiethyleneglycol-succinate (DEGS) coated on 80-100 mesh Diatoport S and an instrument equipped with a hydrogen flame ionization detector. Temperature was programmed from 135 C to 210 C at 2.75 C/min. To aid the identification of peaks not corresponding to available standards, the methoxy bromomercuric adducts of the unsaturated acids were prepared and separated by thin layer chromatography (TLC) into groups containing the same number of double bonds (22).

Fatty acid identification was confirmed by combined gas chromatography-mass spectrometry. The values for the fatty acid distribution of EPG obtained after isolation of EPG by direct TLC fractionation of the lipid extracts were virtually identical to those obtained after purification of EPG by combined DEAE column (23) and TLC procedures.

TABLE III

Brain Total Lipid Concentration (mg/g fresh weight) of Control and EFA Deficient Rats[a]

Days	Control	Deficient
180	8.95	8.35
365	11.50	9.40

[a]The values are determined on the lipid extracts from pooled brains.

Preparation of Myelin

Myelin was purified by ultracentrifugation according to the procedure described by Autilio et al. (24). The myelin pellet obtained was subjected to an osmotic shock and samples were submitted to electron microscopic examination in order to check their purity. They myelin pellet was lyophilized and the lipid extracted. Ethanolamine phosphoglyceride was isolated and its fatty acid methyl esters prepared, purified and analyzed as previously described.

RESULTS

Effect on Body and Brain Growth

A considerable reduction in the rate of body and of brain growth is induced by EFA deficiency as shown in Table I. The values reported are for male animals only but the results obtained with female animals are virtually identical. Brain weights are significantly reduced as early as at 10 days of age in the deficient animals, while body weight is reduced in respect of control values after 60 days. The dietary EFA deficiency thus influences brain growth in a relatively short time.

Brain Phospholipid and Total Lipid Concentrations

Brain phospholipid concentrations are also reduced after 90 days of EFA deficiency, as shown in Table II. This reduction also involves brain total lipids as shown in Table III, which presents the values for purified brain total lipid concentrations in 180- and 365-day-old control and deficient rats.

Brain EPG Fatty Acids

The fatty acid distribution of brain EPG in control and EFA deficient rats at various ages is shown in Table IV. The values for individual fatty acids are not reported so that presentation of the data can be simplified. During normal development, saturated and polyenoic fatty acids tend to decrease while monoenes increase considerably. In EFA deficient animals saturates are scarcely affected. Monoenes remain practically at the same level in both groups up to 90 days, but they tend to decrease in the EFA deficient rats later on. Polyenes are above control values in the treated animals after 60 days of age.

Table V shows the distribution of polyenoic acids of brain EPG of control and deficient rats at various ages. In control rats, dienes and trienes are minor components and remain con-

TABLE IV

Brain EPG Fatty Acid Composition (wt. %) in Control (C) and EFA Deficient (D) Rats

	Saturates		Monoenes		Polyenes	
Days	C	D	C	D	C	D
3	38.0	36.8	11.0	9.6	49.7	52.2
10	38.3	39.8	11.9	9.9	48.3	48.7
30	31.4	29.5	22.2	25.2	46.1	42.6
60[a]	(6)[b] 30.0 ± 1.1	(5) 28.0 ± 1.1	31.1 ± 0.6	32.8 ± 1.1	38.6 ± 1.3	38.9 ± 1.6
90	27.5	23.9	33.3	33.6	39.1	42.6
180	22.3	21.5	39.9	33.0	37.6	45.5
365	31.7	30.4	38.3	33.7	29.6	35.5

[a]These values represent the average of determinations ±SE performed on the EPG fatty acids of individual animals.

[b]The number of animals used is shown in parentheses.

stantly very low, tetraenes decrease slowly and pentaenes (mainly 22:5 w6), which are about 6% of total EPG fatty acids at three days, practically disappear after six months. Hexaenes do not vary appreciably and are slightly decreased only after one year. EFA deficiency enhances trienes (mainly 20:3 and 22:3 w9) and decreases tetraenes (mainly 20:4 and 22:4 w6) as early as at 30 days of age. These changes become prominent in the 6-month- and 1-year-old deficient animals. Pentaenes do not decrease with age in the deficient rats as they do in controls, but remain at a 4-5% level, while hexaenes are reduced, relative to control values, only after six months.

The fatty acid changes in relation to age, in control and deficient rats are summarized in Table VI, which presents the distribution of brain EPG fatty acids in the three families of oleate (w9), linoleate (w6) and linolenate (w3). During one year of normal development, w9 acids (mostly monoenes with 18 and 20 carbon atoms) increase remarkably, w6 acids (mostly 20:4, 22:4 and 22:5) are reduced to about half, while w3 acids (mostly 22:6) do not change significantly. In comparison with control animals, EFA deficiency induces an increase of w9 acids after 30 days, a decrease of w6, which is already evident after 10 days and is prominent after one year, and a reduction of w3 acids only after a long period of time. In spite of these considerable changes, the unsaturation index, which represents the average number of double bonds per fatty acid molecule, remains virtually the same in both groups. This shows a remarkable ability of the molecules to maintain a certain level of unsaturation, regardless of the dietary EFA content.

The severity of EFA deficiency is indicated by the triene-tetraene ratios, which are shown in Figure 1 for control and deficient rats at various ages. This ratio remains very low in control rats (about 0.03) while in the deficient rats it rises rapidly after 10 days and reaches the value of 1.95 after one year in an almost linear progression.

Effects of Control Diet After a Period of Deficiency

The fatty acid distribution of brain EPG in control (C) and EFA deficient animals (D) and in those which, after a period of deficiency, were put on the control diet (D+C) is shown in Table VII. This table shows that feeding the control diet to EFA deficient rats does not appreciably modify the content of saturates and monoenes when compared with controls, but induces a slight but consistent elevation of polyenes. This elevation results in higher values of the unsaturation indices in these groups of animals. Table VIII shows the distribution of polyenoic fatty acids of brain EPG in the same groups of animals. Trienes return to normal values in animals put on the control diet after a period of deficiency. Tetraenes are at a slightly but consistently higher level in deficient rats put on the control diet, showing a

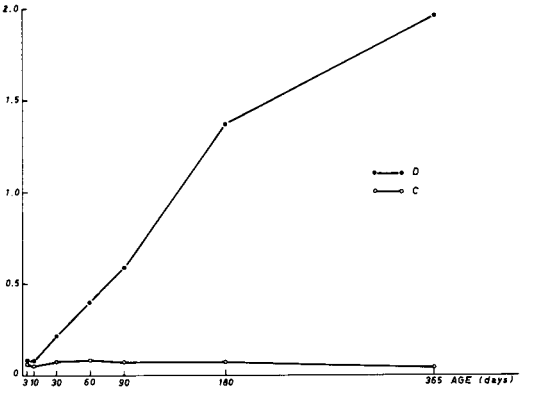

FIG. 1. Trienes:tetraenes ratio in control (C) and deficient (D) rats at various ages.

"rebound" phenomenon. Pentaenes are enhanced over control values in the 30- and 60-day-old rats fed the control diet after a period of deficiency.

In the 180-day-old rats put on a control diet after 90 days of EFA deficiency (90 D + 90 C), pentaenes remain close to the level found in the deficient group, while they are practically absent in controls, and hexaenes are close to control values, while they are reduced in the deficient group. The ratio between trienes of the w9 (derived from oleic acid, 18:1 w9) and w6 (derived from linolenic acid, 18:2 w6) fatty acid families in these groups of animals is shown in Figure 2. This ratio, which gives an indication of the metabolic balance between the w9 and w6 fatty acid families, is approximately 1 at three days of age and remains approximately 0.5 in control animals, while in the deficient rats it increases to 8 or 9 after six months. In animals put on the control diet after an initial period of deficiency, this ratio always remains much higher than the normal values. This is particularly evident in the 180-day-old rats maintained for 90 days on the deficient diet and then fed the control diet.

The fatty acid changes of deficient animals returned to the control diet are summarized in Table IX, which shows the values for the three major fatty acid families of brain EPG in these groups of rats. Giving the control diet after a period of deficiency normalizes the values of w9 acids, which are increased during EFA deficiency, and enhances the levels of w6 acids over the control values.

EPG Fatty Acid Changes in Myelin

The fatty acid distribution of EPG in myelin

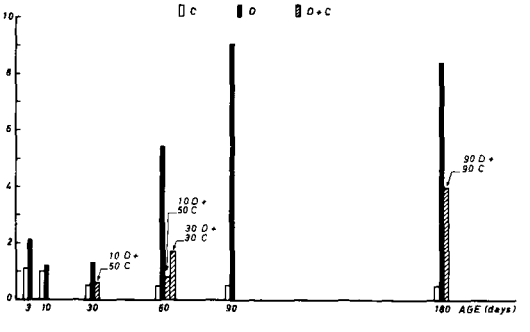

FIG. 2. w9:w6 trienes ratio in control (C) and deficient (D) rats plus deficient rats put on control diet (D+C) at various ages. 10D+20C = 10 days on the deficient diet plus 20 days on the control diet; 10D+50C = 10 days on the deficient diet plus 50 days on the control diet; 30D+30C = 30 days on the deficient diet plus 30 days on the control diet; 90D+90C = 90 days on the deficient diet plus 90 days on the control diet.

TABLE V

Distribution of Polyenes Expressed as wt. % of Brain EPG Fatty Acids in Control (C) and Deficient (D) Male Rats[a]

Fatty acids	3 Days C	3 Days D	10 Days C	10 Days D	30 Days C	30 Days D	60 Days[b] C (6)[c]	60 Days[b] D (5)	90 Days C	90 Days D	180 Days C	180 Days D	365 Days C	365 Days D
Dienes	1.7	1.0	1.2	1.6	1.8	1.8	1.7 ± 0.1	1.5 ± 0.1	1.3	1.0	1.2	1.5	0.4	1.2
Trienes	1.5	2.0	1.4	2.0	1.8	4.2	1.6 ± 0.2	6.4 ± 0.3	1.5	8.9	1.3	15.6	0.7	16.3
Tetraenes	23.9	24.1	25.7	24.0	24.5	19.5	20.1 ± 0.7	16.8 ± 0.5	21.5	15.1	19.0	11.4	16.9	8.3
Pentaenes	6.6	5.1	4.3	3.7	3.6	4.0	3.2 ± 0.2	3.3 ± 0.2	3.6	4.2	0.8	5.2	0.6	4.2
Hexaenes	15.7	20.0	15.7	17.4	13.9	13.1	10.9 ± 0.4	10.5 ± 0.7	11.3	13.1	15.3	11.8	11.0	5.5
Unidentified	1.6	1.6	1.5	1.6	0.6	2.7	0.5 ± 0.1	0.5 ± 0.07	0.5	0.3	0.2	0.3	0.4	0.4

[a]The values represent the average of at least two determinations performed on EPG fatty acids isolated from the pooled brains.
[b]These values represent the average of determinations ±SE performed on EPG fatty acids of individual animals.
[c]The number of animals used is shown in parentheses.

TABLE VI

Brain EPG Fatty Acid Families as wt. % of Total Fatty Acids in Control (C) and EFA Deficient (D) Rats

	w9 acids		w6 acids		w3 acids		UI[a]	
Days	C	D	C	D	C	D	C	D
3	10.6	11.2	30.2	28.3	16.2	18.1	241	259
10	11.2	10.0	29.2	27.6	16.3	17.9	237	238
30	22.0	26.6	28.4	22.5	14.2	14.6	230	218
60	30.7	37.2	23.9	20.2	11.3	10.7	205 ± 16	202 ± 7
90	33.2	40.4	25.7	19.7	11.3	13.4	212	222
180	39.5	48.9	20.5	17.3	15.7	12.0	218	225
365	37.8	49.0	18.5	12.6	11.2	5.8	178	172

[a]UI, unsaturation index: summation of percentage of individual unsaturated fatty acids multiplied by the number of double bonds.

prepared from 180-day-old control and EFA deficient animals and in those that, after a period of EFA deficiency, returned to control diets, are shown in Table X. The fatty acid pattern of rat myelin EPG is considerably different from that observed in whole brain EPG. Concentrations of saturates and polyenes are lower, while those of monoenes are higher in respect to the corresponding values for whole brain EPG. The high content in monoenes is mainly due to the high concentration of C18:1 and C20:1. Tetraenes are lower in myelin than in whole brain EPG, while pentaenes and hexaenes are minor constituents.

EFA deficiency induces a considerable increase of trienes and a corresponding decrease of tetraenes in myelin EPG, which is shown by a rise of the triene:tetraene ratio. The w9/w6 fatty acid ratio is also considerably increased. It can thus be seen that the fatty acid changes induced by EFA deficiency in myelin EPG are very similar to those observed in whole brain.

Return to control diet after 90 days of EFA deficiency reverses the fatty acid changes almost completely, but trienes and pentaenes remain slightly higher and hexaenes lower than in control myelin. The increase of polyenes, especially of the w6 family, observed in whole brain EPG fatty acids in this group of rats, does not appear in myelin EPG.

DISCUSSION

EFA deficiency induced in the rat at the early stage of development affects body and brain growth after even a few weeks of extrauterine life. A reduction in body and brain weights in the offspring from female rats raised to sexual maturity on an EFA deficient diet and then bred has been reported also by Steinberg et al. (15). Early EFA deficiency results in decreased brain phospholipid and total lipid deposition, indicating that dietary polyunsaturated essential fatty acids play a significant role for brain development.

The effects induced by EFA deficiency in the brain consist especially in alterations of the pattern of polyunsaturated fatty acids. The mechanism underlying these changes can be attributed to competitive inhibition in the conversion of different polyunsaturated fatty acids (25). This phenomenon is due to a relative lack of specificity of the enzymes devoted to desaturation and elongation of polyunsaturated acids and to a differential relative affinity of this enzyme system for acids of the oleate, (18:1 w9), linoleate (18:2 w6) and linolenate (18:3 w3) families. This affinity is greater for fatty acids with a higher number of double bonds and thus is higher for linolenate than for linoleate, and is much lower for oleate. Hence the conversion of oleic acid to more unsaturated, longer chain fatty acids, such as w9 trienes, is inhibited by linoleate and linolenate which possess a greater affinity for the enzyme system involved. Conversely, this inhibition is reduced by dietary deficiency of polyunsaturated essential fatty acids.

The changes in the pattern of polyunsaturated fatty acids are particularly evident in EPG, which is the major brain (26) and myelin (27) phospholipid, and is highly unsaturated. EPG fatty acids undergo complex changes during normal development; saturates decrease and monoenes increase. C 16:0 and C 18:0 fatty acids represent 14% and 23% of total EPG fatty acids at three days of age and decrease to 6% and 15% respectively after six months. Conversely C 18:1 and 20:1, which are 10% and 0.3% at three days of age, reach 31% and 9% respectively at the same period of time. These changes are similar to those reported for total rat brain glycerophosphatide fatty acids by Kishimoto et al. (28) and for human brain EPG fatty acids by Svennerholm (29) and Rouser and Yamamoto (30). Polyunsaturates decrease

TABLE VII

Brain EPG Fatty Acid Composition (wt. %) in Control (C) and EFA Deficient (D) Rats and in EFA Deficient Rats Put on Control Diet (D+C)

	30 Days				60 Days			180 Days		
	C	10 D + 20 C	D	C[a]	10 D + 50 C	30 D + 30 C	D[a]	C	90 D + 90 C	D
Saturates	31.4	29.8	29.5	30.0 ± 1.1	28.9	30.0	28.0 ± 1.1	22.3	24.6	21.5
Monoenes	22.2	19.0	25.2	31.1 ± 0.6	29.2	28.0	32.8 ± 1.1	39.9	31.9	33.0
Polyenes	46.1	51.1	42.6	38.6 ± 1.3	41.6	41.9	28.9 ± 1.6	37.6	43.8	45.5
UI[b]	230	251	218	205 + 16	218	216	200 + 7	218	234	225

[a]These values represent the average of determinations ±SE performed on the EPG fatty acids of individual animals. The number of animals is shown in parentheses.
[b]UI, unsaturation index.

TABLE VIII

Distribution of Polyenes Expressed as wt. % of Brain EPG Fatty Acids in Control (C), Deficient (D) and Deficient Switched to Control Diet (D+C) Male Rats[a]

	30 Days				60 Days			180 Days		
Fatty acids	C	10 D+20 C[b]	D	C[c] (6)[d]	10 D+50 C[e]	30 D+30 C[f]	*D (5)	C	90 D+90 C[g]	D
Dienes	1.8	1.9	1.8	1.7 ± 0.1	1.6	1.5	1.5 ± 0.1	1.2	1.0	1.5
Trienes	1.8	2.0	4.2	1.6 ± 0.2	1.6	2.2	6.4 ± 0.3	1.3	2.2	15.6
Tetraenes	24.5	26.2	19.5	20.1 ± 0.7	22.0	21.5	16.8 ± 0.5	19.0	21.4	11.4
Pentaenes	3.6	4.2	4.0	3.2 ± 0.2	5.2	5.1	3.3 ± 0.2	0.8	3.8	5.2
Hexaenes	13.9	16.1	13.1	10.9 ± 0.4	11.2	11.3	10.5 ± 0.7	15.3	15.1	11.8
Unidentified	0.6	0.7	2.7	0.5 ± 0.09	0.3	0.5	0.5 ± 0.07	0.2	0.3	0.3

[a]The values represent the average of two determinations performed on EPG fatty acids from the pooled brains.
[b]10 D+20 C = 10 days on the deficient diet and 20 days on the control diet.
[c]These values represent the average of determinations ±SE performed on the EPG fatty acids of individual animals.
[d]The number of animals used is shown in parentheses.
[e]10 D+50 C = 10 days on the deficient diet and 50 days on the control diet.
[f]30 D+30 C = 30 days on the deficient diet and 30 days on the control diet.
[g]90 D+90 C = 90 days on the deficient diet and 90 days on the control diet.

TABLE IX

Brain EPG Fatty Acid Families in Control (C) and EFA Deficient (D) Rats and EFA Deficient Rats Put on Control Diet (D+C)

	30 Days			60 Days			180 Days		
	C	10 D+20 C	D	C	10 D+50 C	30 D+30 C	D	C	90 D+90 C	D
w9 Acids	22.0	19.0	26.6	30.7	29.2	28.8	37.2	39.5	32.7	48.9
w6 Acids	28.4	30.4	22.5	23.9	27.4	26.1	20.2	20.5	24.4	17.3
w3 Acids	14.2	16.6	14.6	11.3	11.5	11.6	10.7	15.7	15.8	14.5

[a]Values are expressed as percentages of total EPG fatty acid.

slowly with age as seen by the reduction of w6 acids, particularly of 22:5 w6 which represent about 6% of EPG fatty acids at birth and practically disappear six months later. Similar findings are reported (29) for human brain EPG fatty acids. The alterations induced by EFA deficiency in our experimental conditions appear rather early. Steinberg et al. (15) have also shown that fatty acid changes are present in the offspring of female rats raised from birth on an EFA deficient diet. These changes are similar to those reported in other tissues (9,31), and consist mainly in an increase of trienes (C 20:3 and C 22:3 w9) and a decrease of tetraenes. The triene-tetraene ratio is correspondingly already increased at one month of age, i.e. at the end of the lactating period, in the deficient rats.

Monoene levels are not greatly modified in the brains of young deficient rats, as observed by Mohrhauer and Holman (5), while they increase in the liver (32,33). However, monoenes of brain EPG decrease during prolonged EFA deficiency, suggesting reduced availability of brain oleic acid for the conversion to trienes, and, consequently, greater utilization of the in situ sources of this acid.

Pentaene levels, mainly 22:5 w6, are considerably modified after a long period of EFA deficiency. The acid 22:5 w6 is practically absent in 6-month-old control rats, whereas it reaches the value of 5% of EPG fatty acids in the deficient ones. The persistence of this acid may be due to increased conversion of the precursor 22:4 w6, or more likely, to reduced degradation of 22:5 w6. This interpretation is based on the following observations: the turnover of polyunsaturated fatty acids is lower in EFA deficiency (34) since the animal seems to try to preserve its stores of EFA (35,36); on the other hand, fatty acids of the w6 family are preferentially converted to the longer chains, i.e., more unsaturated homologues, rather than catabolized, (37); and 22:5 w6 appears to be the last member of the w6 family (38,39). These observations suggest that elevation of 22:5 w6 of brain EPG during prolonged EFA deficiency is based upon reduced degradation of the last member of w6 fatty acid family. Further investigation on the metabolic fate of brain 22:5 w6 is in order.

The fatty acid changes described for total brain EPG during EFA deficiency are present also in myelin EPG. The modifications induced by EFA deficiency chiefly involve structural lipids, as expected, considering the structural role of phospholipids, such as EPG, containing polyunsaturated fatty acids derived from EFA. However, it is important to recognize that fatty

TABLE X

Fatty Acid Distribution (wt. %) of EPG in Myelin of 180-Day-Old Male Rats

Fatty acids	Control, six months	Deficient and control, three months each	Deficient, six months
Saturates	13.5	13.9	13.5
Monoenes	64.0	63.9	60.1
Polyenes	22.2	21.8	25.6
Dienes	1.8	1.8	1.9
Trienes	2.5	4.2	13.0
Tetraenes	13.9	12.8	7.3
Pentaenes	0.5	1.1	1.6
Hexaenes	3.5	2.0	1.8
w9 acids	63.4	65.7	70.6
w6 acids	16.7	15.3	9.9
w3 acids	3.7	2.2	2.0
Unsaturation index	154	148	151
Triene-tetraene	0.18	0.33	1.74
w9/w6 Trienes	0.24	2.72	9.00

acids deriving from both the monoacyl (plasmalogen) and the diacyl forms of EPG were analyzed, while the saturated ether compound was ignored. The proportion of EPG plasmalogen is much higher in myelin than in whole brain (40), hence myelin data were obtained on a somewhat different fatty acid population.

When EFA deficient rats are given the control diets the levels of trienes are completely normalized. The ratio of the slowly metabolized w9 trienes (3) to the rapidly converted w6 (41) trienes is higher in the above two groups of rats than in control animals. This indicates that the metabolic balance between w9 and w6 fatty acid families is not completely reached even after a long period of feeding a control diet to rats maintained in EFA deficient conditions for an equally long period of time.

In rats fed the control diet after a period of deficiency, an increase of polyenes, especially members of the w6 family, above control values is also observed. This rebound phenomenon in the incorporation of w6 acids, which is depressed during EFA deficiency, is consistent with the observation of an increased incorporation of 1-^{14}C linoleic acid into w6 polyunsaturates (42), together with reduced turnover of the formed PUFA (34) in EFA deficient rats. According to Mead (3), preservation of EFA occurs only after incorporation into structural phospholipids.

This rebound phenomenon of w6 acids does not appear in myelin EPG fatty acids, suggesting a slower turnover of these compounds in more stable structures. However, the considerable reduction of trienes and increase of tetraenes in myelin EPG in the 90 D + 90 C animal group indicates that the turnover of myelin lipids or the exchange of their fatty acids is appreciable even in adult rats.

Another interesting point concerning brain EPG fatty acids of this group of animals (90 D + 90 C) regards the level of pentaene fatty acids. In spite of the prolonged period of return to the control diet, pentaenes remain at a concentration which is much higher than in controls and is close to that observed in the deficient group of the same age. Impairment of pentaene metabolism, induced by a long period of EFA deficiency, is therefore retained even after replacement of the diet.

In conclusion, early EFA deficiency induces considerable alterations in brain structural lipids which may result in changes in structure and function of nervous cells. These biochemical alterations are not completely reversed, even after a prolonged period of return to normal diet.

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Effect of EFA Deficiency on Lipid Transport From Liver[1]

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ABSTRACT

Studies are reported of the effect of an essential fatty acid (EFA) deficiency on synthesis of triglycerides (TG) and phospholipids (PL) and secretion of these compounds by livers of male Sprague-Dawley rats. Animals were fed a semi-purified diet containing corn oil or hydrogenated coconut oil (HCO) as the sole source of fat or no fat from weaning to 20 weeks of age. Liver function of the animals in each group was compared by an isolated liver perfusion technique with perfusates containing erythrocytes and linoleate, and in vivo experiments via tail vein injection of palmitate-^{3}H. Perfusion experiments showed that an EFA deficiency reduced the ability of the liver to secrete TG and PL. Accumulation of TG in the liver and its diminished secretion into the blood of EFA deficient animals were demonstrated by in vivo experiments with palmitate-^{3}H. The rate of conversion of linoleate to arachidonate and synthesis of PL was greater in livers of EFA deficient rats than in the control, corn oil fed animals. The results suggest a relationship of EFA metabolism to lipid transport.

INTRODUCTION

Although accumulation of fat in the livers of essential fatty acid (EFA) deficient animals has been observed by a number of investigators (1-3), the relationship of essential fatty acids to lipid transport from liver has yet to be elucidated. Holman and Peifer (4) and Alfin-Slater et al. (1) speculated that essential fatty acids are important in the mobilization or transport of lipid. However, Sinclair and Collins (5) who reported the accumulation of 9% to 10% fat in the livers of EFA deficient fats concluded there was no impairment of the secretion of triglycerides (TG) from the livers of their animals on the basis of experiments with Triton. In a recent paper (6) we showed via isolated liver perfusion experiments that the capacity of the livers of rats to secrete TG was diminished by an EFA deficiency. Further studies on the accumulation and secretion of TG from the livers of animals fed corn oil or EFA deficient diets via isolated liver perfusion, using perfusates containing erythrocytes, and in vivo tracer experiments with palmitate-^{3}H are reported here.

MATERIALS

Animals

Weanling male rats of the Sprague-Dawley strain (obtained from Dan Rolfsmeyer Co., Madison, Wisconsin) were placed in individual cages and fed ad lib. for 20 weeks a fat free diet or this diet supplemented with hydrogenated coconut oil (HCO) or corn oil in place of an equal weight of sucrose. The fat free diet consisted of, by weight, 29% vitamin test casein, 61% sucrose, 4% salt mixture (Wesson modified, Obsourne-Mendel salt mix: General Biochemicals, Chagrin Falls, Ohio.), 4% cellulose (Non-nutritive cellulose Alphacel: Nutritional Biochemicals Corporation, Cleveland, Ohio.), 1% casein containing vitamins in the required amounts (7) and 1% choline mixture (22% choline dihydrogen citrate, 78% casein). Linoleic acid-1-^{14}C (sp. Act. 52.9 mc/mM) and palmitate-9,10-^{3}H (sp. Act. 100 mc/136 mg) were obtained from Nuclear Chicago and New England Corp., respectively. These labeled compounds were purified to >95% by thin layer chromatography. Linoleic acid, >99% purity, was purchased from The Lipids Preparation Laboratory of The Hormel Institute.

METHODS

Liver Perfusion

Livers were perfused by essentially the same technique as described by Ruderman et al. (8), using a double unit modified Miller apparatus (Metaloglass Inc., Boston, Mass., Model 81-68). The basic perfusion medium consisted of 200 ml of Krebs-Ringer bicarbonate buffer (100 ml for each apparatus) containing approximately

[1]One of five papers to be published from the Symposium "Lipid Transport" presented at the AOCS Meeting, New Orleans, April 1970.

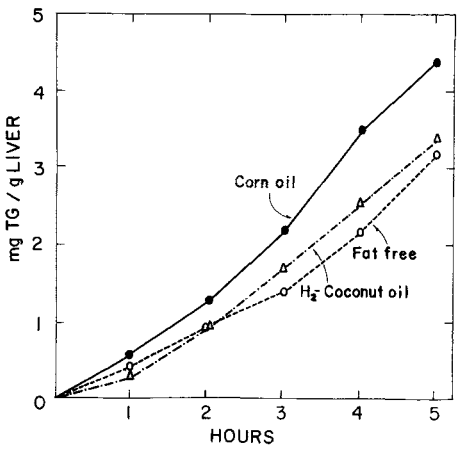

FIG. 1. Release of TG from liver during perfusion with simultaneous infusion of 575 μmoles of linoleate for 5 hr. Each point represents the average of four animals in fat-free, two in hydrogenated coconut oil and five in corn oil groups.

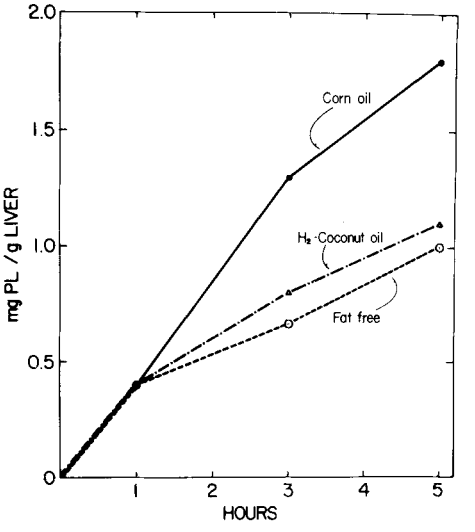

FIG. 2. Release of PL from liver during perfusion with simultaneous infusion of 575 μmoles of linoleate for 5 hr. Each point represents the average of two experiments in each group.

24% erythrocytes and 4% purified albumin. The bovine serum albumin (Fraction V powder, Eastman Organic Chemicals) was treated with charcoal (9) to remove free fatty acids and dialyzed against physiological saline (10). Erythrocytes were isolated from fresh bovine blood as described by Exton et al. (11). Livers were prepared for perfusion as previously described (6) except that an 8 to 10 cm cannula was inserted into the inferior vena cava in order to maintain normal outflow of perfusate and to avoid edema (11). In most experiments livers were perfused in pairs, one from the corn oil group which served as a control and the other from either the HCO or fat free groups for direct comparison with the same perfusate. Flow rate of perfusate was maintained at 30 ml/min. Sodium linoleate (575 μmoles, in several experiments containing 12 μc of 1-^{14}C-linoleate) was prepared by neutralization of the acid with ethanolic sodium hydroxide. A small amount of antioxidant (0.01% NDGA w/w of linoleate) was added to this solution to prevent autoxidation during perfusion. The linoleate solution was diluted to a final volume of 30 ml with water and infused into the perfusate over a 5 hr period with an infusion pump (Model 1100, Howard Apparatus Co., Inc., Mills, Mass.). In several experiments a partial lobectomy of the liver was performed before the perfusion was started to obtain lipid for fatty acid analysis.

In Vivo Experiments

Animals of each group were anesthetized by intramuscular injection of 5 mg/100 g of body weight of sodium pentobarbital and injected in the tail vein with 10 μc of palmitate-^{3}H in rat serum (12). The animals were maintained at 37 C during the experiment. At periodic intervals a small samples of liver (approximately 0.5 g) was excised and removed through a small incision in the upper abdomen. Approximately 1 ml of blood was also taken at selected intervals from the retroauricular plexus. Total plasma volume was calculated as described by Sinclair and Collins (5).

Analysis of Lipids

Lipid was extracted according to the method of Folch et al. (13), and TG, phospholipids (PL) and fatty acid composition were determined as previously described (6). Results were expressed as mean ± standard error. Student's t test or a modified formula to take into account large differences in variances (14) was used for statistical analysis.

RESULTS

At the end of 20 weeks the TG levels of the livers of the animals of the three groups, fat free, HCO and corn oil were 19.7±3.4 [8], 14.1±0.5 [7] and 9.9±0.9 [10] mg/g liver, respectively, (the number in brackets is the number of animals for which individual values were obtained). The level of the TG in the livers of the EFA deficient animals were slightly but significantly higher than those of the corn oil

TABLE I

Per Cent Incorporation of Radioactivity Into Liver Lipids and Perfusate TG

	Corn oil	Fat free	Hydrogenated coconut oil
Liver PL[a]	14.1±0.6[b] (3)[c]	17.6±1.3 (3)	16.5 (2)
Liver TG[a]	25.4±3.9 (3)	20.4±3.3 (3)	18.0 (2)
Perfusate TG	12.5±3.2 (3)	5.4±0.4 (3)	5.8 (2)
Ratio of perfusate TG to liver TG	0.51±0.03 (3)	0.28±0.04[d] (3)	0.32 (2)

[a]Abbreviations: Ph, Phospholipids; TG, Triglycerides.
[b]Mean ± SE.
[c]Number of animals.
[d]$P < 0.05$ to corn oil group.

groups.

Liver Perfusion Experiments

The rates of secretion of TG from the livers of animals of the three groups during perfusion experiment is shown in Figure 1. These results demonstrate the generally slower rate of secretion of TG from the livers of the EFA deficient than the corn oil group of animals. The generally slower rate of release of PL from the livers of the EFA deficient animals is illustrated in Figure 2. Amounts of TG in perfusate after 3 hr perfusion of corn oil, fat free and HCO oil groups were 2.1±0.3 [5], 1.3±0.3 [4] and 1.8 [2] mg/g liver, respectively, and the difference between the corn oil and the fat free group was significant ($p < 0.05$) on paried analysis. Amounts of PL in perfusate after 3 hr perfusion of corn oil, fat free and HCO groups were 1.1±0.1 [4], 0.7±0.1 [4] and 0.8 [2] mg/g liver, respectively, and the difference between the corn oil and the fat free group was also significant ($p < 0.05$).

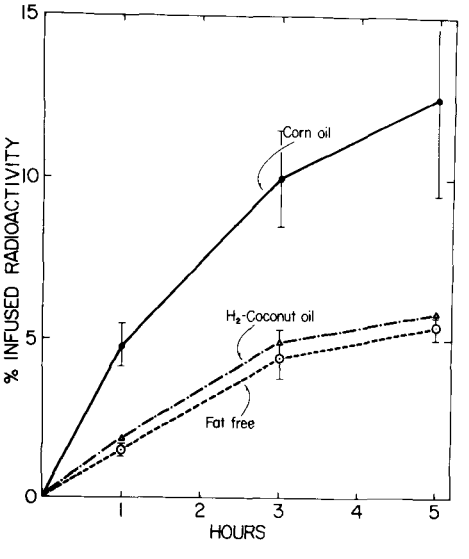

FIG. 3. Per cent incorporation of radioactivity into perfusate TG during 5 hr liver perfusion with simultaneous infusion of 575 μmoles of linoleate containing 12 μc of 1-^{14}C-linoleate.

Per cent incorporation of radioactivity in experiments with linoleate-1-^{14}C (Figure 3) also shows that less TG is secreted into the perfusates of the EFA deficient animals than in those of the corn oil group. The values were not significantly different at the end of the experiment because of the large variation in the corn oil group. However, the differences between the fat free group and the corn oil group are significant at the 1 and 3 hr periods. In several experiments the distribution of TG secreted into the perfusate in the form of β-lipoprotein and the other lipoprotein fraction was determined by the method of Burnstein et al. (15). Both mass analysis and distribution of radioactivity showed that more than 90% of the TG was present in the perfusate as β-lipoprotein in all three groups.

The per cent incorporation of infused linoleate-1-^{14}C into liver PL was slightly larger and into liver TG slightly smaller after 5 hr of perfusion (Table I) but the differences were not significant. However, the ratio of radioactivity between the perfusate and liver TG was lower in the EFA deficient than the corn oil group. Radioactivity remaining in the FFA fraction of perfusate at the end of the experiment was about 3% to 5% of that infused indicating that infused linoleate has been taken up and utilized equally well by all groups.

Fatty acid composition of the liver PL at the beginning and after 5 hr of perfusion showed an appreciable amount of the infused linoleate was

TABLE II

Fatty Acid Composition and Distribution of Radioactivity in Liver PL[a]

	Time, hr	14:0	16:0	16:1	18:0	18:1	18:2	20:3ω9	20:4
Corn oil	0	0.4[b]	24.0	0.9	25.9	6.5	14.8	—	27.6
Corn oil	5	0.5	22.2	1.2	27.1	6.8	18.3	—	24.1
Corn oil	5	(1.4)[c]	(6.1)[d]		(2.4)	(6.2)	(54.8)	(12.5)[e]	(9.0)
Fat free	0	0.4	21.2	9.5	20.7	22.7	2.6	17.3	5.6
Fat free	5	0.3	16.1	7.4	18.5	22.5	9.8	14.5	10.9
Fat free	5	(0.7)	(5.2)[d]		(2.3)	(4.6)	(29.8)	(10.6)[e]	(24.2)

[a]Figures without parenthesis show the fatty acid composition of liver phospholipid at the beginning and after 5 hr liver perfusion infusing 575 μmoles of linoleate. Figures in parenthesis show the per cent distribution of radioactivity incorporated into liver PL after 5 hr perfusion infusing 12 μc of ^{14}C-linoleate along with 575 μmoles of linoleate.

[b]Average of three animals.

[c]Average of two animals.

[d]Per cent of radioactivity in mixture of 16:0 and 16:1.

[e]Per cent of radioactivity collected between 18:2 and 20:4. Probably this radioactivity consists of a mixture of 18:3, 20:2 and 20:3 of ω6 series.

converted to arachidonate in the liver of EFA deficient animals (Table II). Because the livers of the corn oil fed animals contained a fair amount of arachidonic acid it was not possible to make an estimation of the amount of conversion of linoleate in the livers of these animals by a simple mass analysis. However, the relative amount of radioactivity distributed in linoleate and arachidonate determined by radio GLC (16) showed that linoleate was converted to arachidonate in the livers of the corn oil group at a much slower rate than in the EFA deficient groups in accord with the results of in vitro experiments (17).

Experiments In Vivo

The rate of incorporation of radioactivity into the liver and serum TG injected with palmitate-^{3}H is shown in Figure 4. Rate of incorporation of radioactivity was higher and decreased slower in the livers and was lower in the serum of the animals of the EFA deficient groups than in those of the corn oil group. The results were significant at the 60 min period after injection as illustrated in Table III. Similar results were obtained in one animal of the fat free group and two of the corn oil group in experiments continued for 120 min. At the end of the experiment, the incorporation of palmitate into PL was slightly higher in the livers of the EFA deficient than the corn oil group but the differences were not significant (Table III). Only about 0.3% of injected label was incorporated into serum PL and there was no significant difference between the three groups.

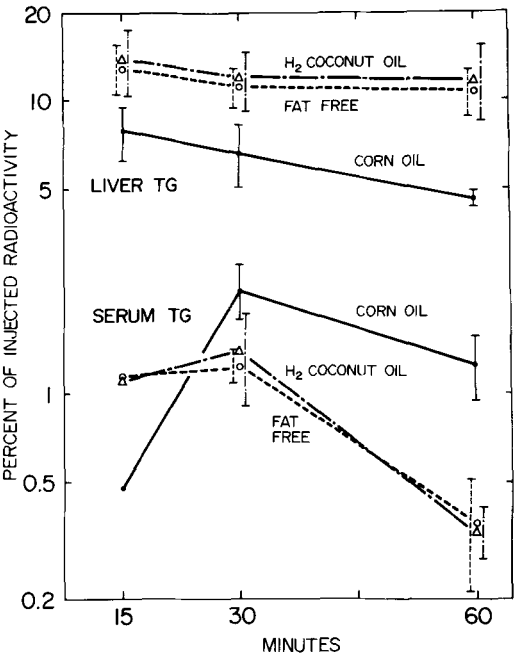

FIG. 4. Per cent incorporation of 10 μc of injected palmitate-^{3}H into liver and serum TG. Each point represents the average of three or more values except the values for serum at 15 min. Values of serum at 15 min are the results of one of each group.

DISCUSSION

Results of the perfusion experiments in this study are in complete accord with those of previous studies (6) using cell free perfusates. The longer experiments in the present study permitted time course relationships to be obtained without the complicating effect of

TABLE III

Per Cent Incorporation of Injected Palmitate-^{3}H Into Liver Lipids and Serum TG, in vivo

	Corn oil	Fat free	Hydrogenated coconut oil
Liver PL[a]	8.6±1.2[c] (5)[b]	9.8±1.5 (4)	13.7±2.7 (3)
Liver TG	4.6±0.3 (5)	10.7±2.0[d] (4)	11.8±3.4 (3)
Serum TG	1.23±0.30 (5)	0.36±0.15[e] (4)	0.34±0.07[e] (3)

[a]Abbreviations: see Table I.
[b]M ± SE.
[c]Number of animals in parentheses.
[d]$P<0.05$ on paired analysis to corn oil group.
[e]$P<0.05$ to corn oil group.

hypoxia. Figures 1,3 and 4 clearly show that the rate of secretion of TG is impaired by an EFA deficiency. In the post absorptive condition, plasma lipid level depends mainly on the balance between release of lipid from the liver and uptake of lipid in peripheral tissue. In the peripheral uptake of lipid, lipoprotein lipase activity in adipose tissue is increased by an unsaturated fat diet (20), and endogenously synthesized lipid appears to be a better substrate for lipolysis via lipoprotein lipase after highly unsaturated fats are ingested (21). Therefore, the cause of low level of plasma TG or very low density lipoproteins in EFA deficient rats (5,18,19) could well be due to impairment in the release of lipid from the liver in accord with the findings reported herein.

The greater accumulation of radioactivity in the TG of the livers of the EFA deficient animals injected with palmitate-^{3}H showed that fatty acids taken up from the blood accumulated in the TG of the livers of the EFA deficient animals to a greater extent than those fed corn oil. Palmitate was selected for use in these experiments because the pool size of this acid is about the same in the liver of fat free and corn oil groups (6). The decreased accumulation of linoleate-1-^{14}C in the liver TG of EFA deficiency during perfusion might be related to increased turnover of PL (22), increased incorporation of linoleate into PL (23) and/or increased oxidation of fatty acids as observed in previous experiments (6).

A number of factors could contribute to the accumulation of fat in the liver of EFA deficient animals. The synthesis of fatty acids is increased by an EFA deficiency (24,25) and could be important under some conditions. Experimentally produced fatty livers (carbon tetrachloride, ethionine or choline deficiency) are believed to be caused by impairment in the synthesis or release of lipoprotein required for lipid transport (26).

A role of EFA in lipid transport may be a requirement for lipoprotein synthesis concerned with lipid or protein. The fact that linoleic acid and apparently arachidonic acid also (because linoleic acid was converted to arachidonic acid) had no immediate effect on triglyceride secretion indicated that the defect involves the synthesis of lipoprotein concerned with structural elements in membrane function. Presumably, these changes require some time to be effected and thus there is no immediate effect of linoleate in the perfusion experiments. Evidence of this effect is that restoration of normal fatty acid composition of liver PL and normal rate of release of protein from the liver in EFA deficient rats requires the feeding of linoleate for several days (27). Restoration of normal swelling properties of liver mitochondria of EFA deficient animals also requires approximately two days of linoleate feeding (28). These observations suggest that the effect of an EFA deficiency on lipid transport probably involves the physicochemical properties of the membranes of the liver cells.

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Studies on the Role of Phospholipids in the Triglyceride Cycle: III. Liver and Plasma Phospholipid Exchange in Depancreatized Dogs[1]

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ABSTRACT

Lipid mobilization from the liver to extrahepatic sites of utilization and to adipose tissue for storage and recycling via the triglyceride cycle requires de novo synthesis of liver lecithins. The role of liver phospholipid (PL) synthesis and plasma phospholipid turnover has been studied under a number of conditions which appear to relate liver lipoprotein formation and release to liver PL synthesis and transport. Conditions which enhance nonesterified fatty acid (NEFA) release from adipose tissue or which inbibit liver PL synthesis prompt liver lipid accumulation. Lipid accumulates in the liver principally as triglyceride when any one of a number of factors required for liver lipoprotein complex formation is blocked, such as by ethionine, or when NEFA release to plasma is increased, such as in cold acclimatization. The relationship of liver PL to liver lipid transport is shown also following recovery from these conditions. Recovery from liver triglyceride (TG) accumulation is accompanied by increased PL synthesis, remobilization of liver PL, and increased turnover of plasma phospholipids. The relative specific activity (RSA) of phospholipid phosphorus (SA of plasma to liver PLP) is increased in depancreatized dogs during liver lipid accumulation. Following the initial depression in liver PL synthesis and transport caused by ethionine administration the RSA increased in all animals studied, indicating a remobilization of the accumulated TG. This remobilization of liver lipid occurs to a greater extent in depancreatized dogs than in normal dogs under the same conditions.

INTRODUCTION

The transport of endogenous fatty acids as triglycerides (TG) or as glycerides has been recognized since the 1957 findings of Carlson and Wadström (1) that TG and some lower glycerides are present in plasma during fasting. However, Fredrickson and Gordon reviewing this work in 1958 (2) point out that the mere presence of glycerides in the plasma during these times does not imply that they are participating in significant net transport of endogenous fatty acids. It was also pointed out in the same review that there were no lines of experimental evidence which would prove an important role of plasma phospholipids in the net transport of fatty acids except as components of the lipoprotein carrying triglycerides in the blood. It was recognized at the time that free fatty acids, or nonesterified fatty acids (NEFA), were released by lipolysis from adipose tissue and circulated to other tissues in combination with plasma albumin (3-6).

Conditions which enhance NEFA release from adipose tissue, or which inhibit liver lipoprotein formation also prompt liver lipid accumulation. The increased liver lipid content under these conditions, classically called "fatty liver," is mainly TG. The pathophysiological etiology of fatty liver is extremely diverse ranging from chronic alcoholism, uncontrolled diabetes mellitus, and certain nutritional deficiencies to a cellular toxicity response to a variety of nonspecific and specific liver toxins. Lipid accumulates in the liver parenchyma principally as TG when any one of a number of factors required for liver lipoprotein formation or for release of liver lipoprotein is blocked, or when NEFA release to the plasma is increased in excess of the energy requirements of the animal. Changes in the rate of removal of TG from the blood may be an important factor in the regulation of liver PL synthesis as appears to be the case in TG transport from the liver to extrahepatic sites (7). Similarly, a fall in hepatic triglyceride could be secondary to a reduced uptake of NEFA by the liver (8). Many studies have concerned the role of hormones, and particularly catecholamines, on the mobilization of lipids. The NEFA release from adipose

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TABLE I

Effects of Pancreatectomy and dl-Ethionine on Blood Glucose and Total Liver Lipids in Female Dogs

Dog No.	Condition	Time after ethionine, min	Blood glucose, mg, %	Total liver lipids, g %
1	Unoperated	0	94	3.89
		0	73	3.98
		0	68	4.34
		60	52	4.27
		150	42	4.54
		300	40	4.56
		390	37	4.94
		450	---	5.35
2	Depancreatized	0	356	13.59
		0	274	15.54
		0	233	16.70
		60	242	15.72
		205	245	16.16
		305	266	17.34
		375	314	17.75
3	Unoperated	0	63	5.15
		60	42	5.12
		150	40	5.47
		270	29	5.63
		360	24	5.60
		540	31	5.62
		615	29	---
4	Depancreatized	0	428	11.80
		105	482	13.20
		330	412	14.34
		420	396	14.82
		600	467	---

tissue is under the combined control of corticosteroids and catecholamines and even an excess of one of these agents induces no stimulation unless a certain concentration of the other is present in the system (9).

While many substances, as well as certain physiological conditions, increase hepatic lipid content, substances which reduce hepatic lipids are known as lipotropic agents. Choline is considered by Greengard (10) to be the most representative of lipotropic agents which, as a constituent of lecithin, is essential for the normal transport of fats acting by enhancing phospholipid turnover and promoting the transport of fat from liver to tissues.

Triglyceride synthesis by the liver occurs primarily by way of glycerol phosphate (11). Experiments by Bjórnstad and Bremer(12) have shown that the terminal reaction in phosphatidyl choline (PC) synthesis is freely reversible since the specific activity of CDP-choline-^{14}C in liver was about equal to the specific activity of liver lecithin-^{14}C. Thus

CDP-choline + diglyceride$\rightleftharpoons$CMP + PC.

Interconversions of phospholipids can occur in liver (13) yielding PC by stepwise methylation of ethanolamine which may be derived from serine by decarboxylation. These reactions can take place while the nitrogenous bases are covalently bound to phospholipids. Bjórnstad and Bremer (12) have shown that while the methylation pathway for PC synthesis occurs predominantly in liver, the PC derived from both pathways enter a common pool which is in equilibrium with plasma.

The work presented here concerns the mechanisms for lipid mobilization from the liver to extrahepatic sites of utilization and storage. The vehicle for this transport is lipoprotein, the water soluble lipid complexes which have been studied extensively and presented in this symposium by Dr. Lopez (14). Results of previously published portions of the present study (15,16) indicated that liver lipid mobilization required de novo synthesis of liver lecithins.

The relative specific activity (RSA) of phospholipid phosphorus (PLP), or specific activity (SA) of plasma to liver PLP, is a measure of the exchange of liver and plasma phospholipids. Because the RSA measurements in the studies

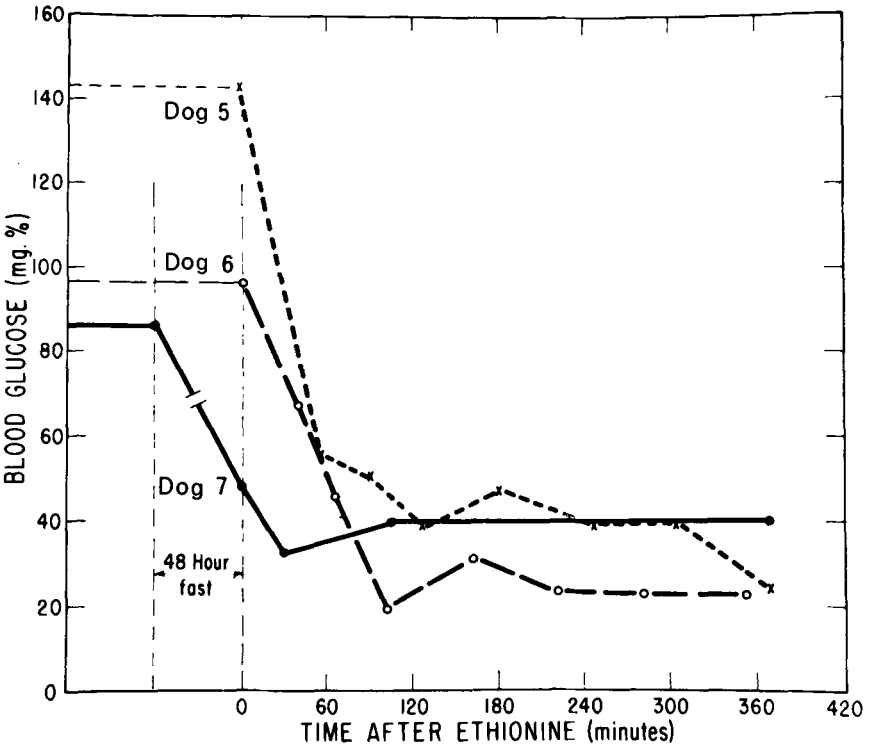

FIG. 1. Blood glucose of dogs after a single oral administration of *dl*-ethionine (1 g/kg).

using rats that were fasted and rats that were given single injections of ethionine indicated a dynamic function of phospholipids in the TG cycle, further experiments were carried out to determine if the RSA would reveal similar effects during periods of rapid liver lipid accumulation irrespective of an acute choline deficiency or ethionine inhibition of choline as a substrate for the synthesis of specific liver lecithins. The depancreatized dog seemed suitable for such a study since it is known that withdrawal of insulin and raw pancreas from the maintenance ration of such an animal leads to the rapid accumulation of liver TG. Ethionine superimposed upon these conditions would enhance the development of a fatty liver and thus exaggerate the predicted exchange phenomenon of liver and plasma PLP under these conditions.

EXPERIMENTAL PROCEDURES

Two female dogs (No. 2 and 4) were depancreatized according to standard procedures (17) and were maintained on insulin and raw pancreas until the time of the experiments in which P^{32}-orthophosphate and ethionine were administered. Two other female dogs (No. 1 and 3) were used as unoperated control dogs. The ethionine dosages and the method of acute administration to the dogs were the same as previously described (16). The P^{32}-orthophosphate was administered intravenously and samples of the blood were taken from the femoral artery at several time intervals after the administration of the isotope while the dogs were under pentobarbital anesthesia. At about the same time that the blood samples were taken, liver biopsy samples were obtained surgically followed by suturing Gelfoam in the wound area to stop bleeding. Analyses for the total liver lipids, and measurement of specific activity of plasma and liver phospholipids were the same as previously described (15,16). Blood glucose was measured by the method of Kemp et al. (18). Three other dogs were used for the measurement of blood glucose after ethionine administration. One dog was given ethionine in the fed state (male dog No. 5) while the other two (male dog No. 6 and female dog No. 7) were given ethionine after a 48 hr fast. The ethionine was administered orally to these animals by force-feeding a gelatin capsule containing the requisite dosage (1 g/kg).

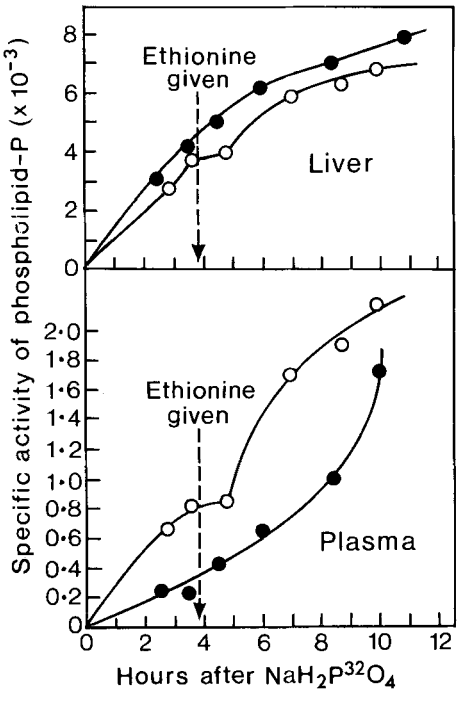

FIG. 2. The specific activity of liver and plasma phospholipid phosphorus before and after *dl*-ethionine in normal (•) and depancreatized (○) dogs. (Dogs 1 and 2).

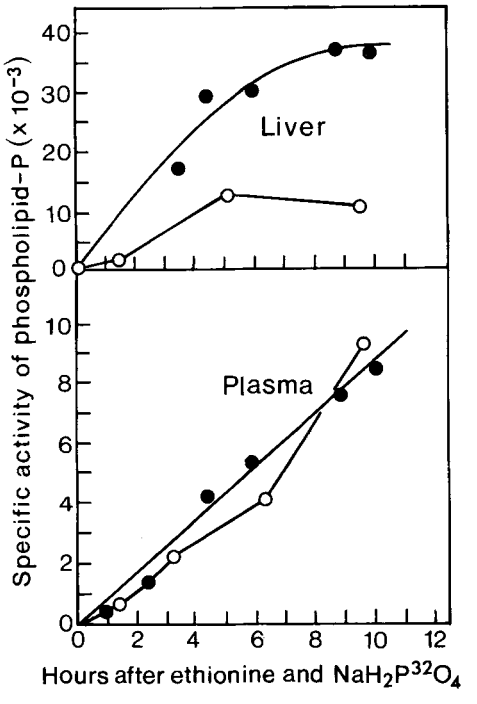

FIG. 3. The specific activity of liver and plasma phospholipid phosphorus in normal (•) and depancreatized (○) dogs given *dl*-ethionine and P^{32}-orthophosphate simultaneously. (Dogs 3 and 4).

RESULTS

The total liver lipids in the depancreatized dogs showed increases of 4.16 and 3.02 g/100 g during the experimental periods, whereas the total liver lipids in the unoperated dogs showed increases of only 1.55 and 0.47 g/100 g. Although the total liver lipids in the depancreatized dogs rose as high as 14.82 and 17.75 g/100 g, compared to 5.35 and 5.62 g/100 g in the unoperated dogs, the liver lipids in the depancreatized dogs were high at the start of the experiments, and hence, the percentage increase in the liver lipids was 39.8%, 30.6%, 9.1% and 25.6% for dogs No. 1-4, respectively (Table I).

The administration of *dl*-ethionine drastically reduced the blood glucose. This was observed in three dogs given a single oral administration of ethionine. Two of the dogs were given ethionine following a 48 hr fasting period and died within 24 hr after ethionine. The other dog was not fasted prior to ethionine administration and it survived for four days. The blood glucose decreased to about the same level in both the fasted and nonfasted dogs (Fig. 1).

A similar blood glucose lowering effect following ethionine was not seen in the depancreatized dogs in which the blood glucose levels were already elevated, nor were any significant changes in blood glucose seen in the unoperated control dogs given ethionine while under pentobarbital anesthesia (Table I). The depancreatized dogs remained hyperglycemic throughout the measurements of plasma and liver PLP specific activities, while the nondiabetic dogs showed only slight lowering of blood glucose during this period.

The uptake of P^{32}-orthophosphate into liver and plasma PL measured at 3 and 4 hr prior to the administration of ethionine and at several time intervals after ethionine, showed a transient inhibition in the depancreatized dog and no apparent effect in the unoperated dog (Fig. 2).

When ethionine was given to the experimental animals at the same time that the P^{32}-orthophosphate was administered, the uptake into liver PLP was found to be lower in the depancreatized dog than in the unoperated control dog. The amount of this difference was greater than that observed in the preceeding experiment (Fig. 3).

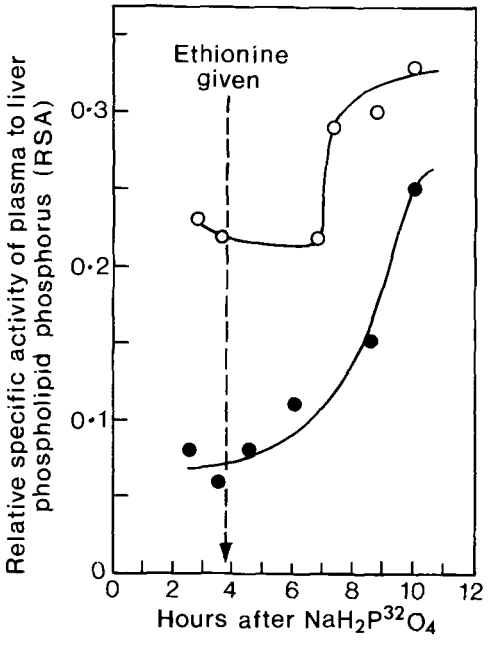

FIG. 4. The relative specific activity of plasma to liver phospholipid phosphorus before and after *dl*-ethionine in normal (•) and depancreatized (○) dogs. (Dogs 1 and 2).

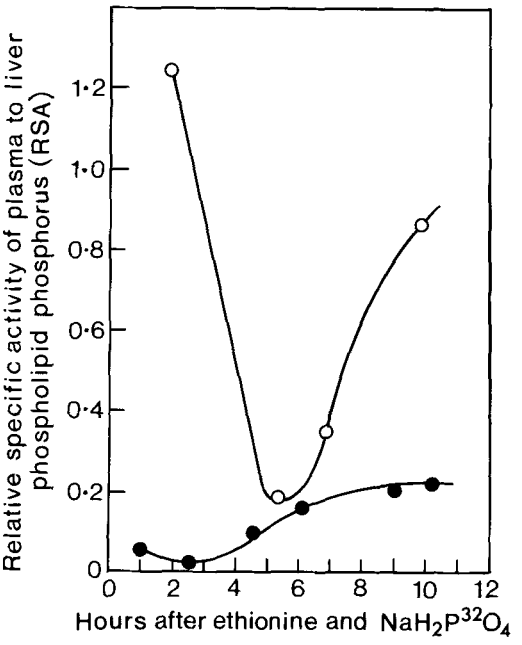

FIG. 5. The relative specific activity of plasma to liver phospholipid phosphorus in normal (•) and depancreatized (○) dogs given *dl*-ethionine and P^{32}-orthophosphate simultaneously. (Dogs 3 and 4).

While the magnitude of the temporary inhibition of liver and plasma PLP synthesis was not very great when ethionine was given during the time course of P^{32} orthophosphate uptake, the effects on the RSA are quite significant. At early intervals, the RSA values for the depancreatized dogs were higher than the corresponding values for the nondiabetic dogs. After ethionine administration the RSA value decreased temporarily in the diabetic dog (Fig. 4).

When the ethionine was given at the same time as the P^{32}-orthophosphate, the decrease in RSA was not observed until the SA of liver PLP was approaching the maximum SA (Fig. 5).

During the later time intervals after P^{32}-orthophosphate administration the RSA increased in all four animals. The increase occurred more abruptly and to a greater extent in the animals given ethionine after the administration of the radioactive phosphate and the effect was greater in both depancreatized dogs than in the nondiabetic dogs.

DISCUSSION

The value of the RSA measurement in estimating lipid transport from the liver is apparent from the findings reported here. The RSA values observed under the conditions of these experiments show changes relating first, to an increased RSA in the diabetic dog, second, relating to the inhibitory effect of ethionine on liver PL formation at early time intervals after ethionine administration, and finally, relating to the remobilization of liver PL after the ethionine had depleted. The remobilization was greatest in the depancreatized dogs. While an increase in the RSA at later time intervals after the administration of ethionine occurred in all animals tested, the effect was more pronounced in the diabetic dogs that were accumulating greater amounts of liver lipid during the time of measurement.

In rats (15) ethionine was shown to cause inhibition of liver phospholipid mobilization to the plasma accompanied by an increase in liver TG. The effect appeared to occur to a greater extent in animals which developed a more intense fatty liver. Changes in liver lipid mobilization as a function of time after ethionine suggested remobilization of liver PL just before or at about the same time that de novo synthesis of liver PLP would be back to normal levels. Changes in the RSA during the course of fasting suggested increased mobilization of liver PL at times when liver TG cycling would be enhanced. In normal dogs (16), ethionine blocks

the turnover of plasma PL and the transport of phospholipids from liver to plasma. Ethionine stopped the disappearance of native labeled plasma PL (nPLP^{32}) abruptly, indicating that some immediate effect of ethionine, perhaps on choline metabolism, was responsible for altering the uptake of plasma PL by the peripheral tissues.

It would appear that the mechanism by which ethionine blocks the transport of phospholipids, and hence the release of lipoproteins to the plasma, is by blocking synthesis of liver lecithins. When this effect of ethionine "wears off," remobilization of liver PL to the plasma occurs as evidenced from the increase in the RSA of plasma to liver PLP in the present study.

The data presented here show that ethionine decreases the uptake of radiophosphate into liver and plasma phospholipids, but that the effect is transient in nature. In these experiments such inhibition of liver PL synthesis must be evaluated by changes in the slope of the uptake curves before and after ethionine administration since a continual uptake of radiophosphate into liver and plasma PLP is observed as a function of time after radiophosphate administration. This was most apparent in the liver inhibition of PL synthesis by ethionine which occurred to a greater extent in depancreatized dogs which accumulated greater amounts of total liver lipid than the nondiabetic control dogs.

It is most important to note that the RSA was decreased by ethionine during the early intervals after administration, and was increased at later time intervals after administration, i.e., near the time of the SA_{max}. The fact that PL exchange occurs to a greater extent in depancreatized dogs than in the unoperated controls suggests that an increased exchange of liver and plasma PL is related to the accumulation of total liver lipids. Animals that show increased hepatic TG accumulation after ethionine administration also show a significant increase of RSA of plasma to liver PLP. Increased hepatic TG, therefore, appears to be a stimulus to remobilization of liver lipid.

The increased exchange of phospholipids seems to be unrelated to the glucose levels in blood since blood glucose was high in the depancreatized dogs, and low in the unoperated controls. Earlier observations (19) show low blood glucose after ethionine in both male and female rats where a pronounced difference in hepatic TG accumulation is seen; the females got a fatty liver whereas the males did not. Although the apparent availability of carbohydrate in the blood of depancreatized dogs would seem to argue against the conclusions that carbohydrate depletion can by itself give rise to increased lipid mobilization for oxidative purposes, it should be mentioned that increased blood glucose in the diabetic condition is secondary to the insulin deficiency which limits substrate oxidation of both carbohydrates and lipid.

Studies on the turnover of plasma PL in depancreatized dogs (20) and in dogs after phlorizin (21) show an increased RSA of liver to plasma PLP which the authors relate to an increased oxidative metabolism of lipids. Robinson and Seakins have shown that there is a reduction in the rate of formation of plasma lipoprotein in the early stages after giving ethionine or puromycin resulting in accumulation of liver TG (22-24). These authors suggest that impaired release of lipoprotein from the liver to the plasma results in hepatic TG accumulation. Although PL metabolism and lipoprotein release are important in regulating liver TG content and output, other possible mechanisms must be considered, notably the effects of ethionine on liver S-adenosyltransferase reactions effectively reducing the availability of adenosine triphosphate. This area, and other considerations of fatty liver induction by ethionine, has been thoroughly reviewed by Farber (25).

The conditions of the present experiments allowed the measurements of plasma and liver phospholipid exchange to be made during the time when there is a very rapid accumulation of hepatic TG. It is interesting to note that although the administration of ethionine affected the time course of radioactive phosphate uptake into phospholipids only transiently, the effects on the exchange of liver and plasma phospholipids persisted longer, and that subsequent to the depression of RSA there was a marked and persistent rise in PL transport especially in those animals which had a fatty liver.

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The Phospholipids of Corynebacteria

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ABSTRACT

The phospholipids of *Corynebacterium diphtheriae*, *Corynebacterium xerosis*, *Corynebacterium equi* and *Corynebacterium ovis* were examined, largely by chromatographic procedures. In all of these, lipids of the phosphoinositide and mannophosphoinositide type were prominent. In contrast to the mycobacteria, the mannophosphoinositides of the corynebacteria were all dimannophosphoinositides; however, as in mycobacteria, these dimannophosphoinositides apparently occurred in the diacylated and triacylated forms—the tetraacylated component prominent in mycobacteria was absent. Phosphatidylethanolamine and phosphatidylserine were also absent. In *Corynebacterium diphtheriae* the major single phospholipid corresponded to phosphatidylglycerol: cardiolipin also appeared to be a major lipid. The fatty acids of the corynebacterial phospholipids were distinguished by the presence of branched chain isomers of medium chain length. The importance of phospholipids in the taxonomy of the actinomycetes and related eubacteria is discussed.

INTRODUCTION

The close phylogenetic relationship of mycobacteria, corynebacteria, propionibacteria and nocardia (1) is supported by morphological (2) and immunological evidence (3). For certain members of these genera, the relationship is also shown by studies on the infrared absorption spectra of whole cells (4), by the presence of complex α-branched β-hydroxylated fatty acids (5) attached to either trehalose (6), glucose (7) or cell-wall arabinose (8), and the presence of large amounts of free trehalose (9) and a typical cell wall mucopeptide (10). Moreover, there is evidence that some corynebacteria contain mannophosphoinositides (11,12) previously thought to be restricted to the mycobacteria. In the present work we are concerned with this latter relationship.

The wide spectrum of phosphoinositides of mycobacteria have been extensively examined by Ballou and coworkers. They range from the simplest lipid, 1-phosphatidyl-L-myoinositol, through those with from one to six molecules of mannose glycosylated to the myoinositol ring (13). The predominant component contains two mannose units (14) and was regarded as 1-phosphatidyl-L-myoinositol 2,6-di-O-α-D-mannopyranoside. However, this dimannoside is now known to exist in several acylated forms, the major ones containing two, three and four molecules of fatty acid (15,16).

Previously the morphological relationships between the propionibacteria and mycobacteria were discussed and their close relationship was substantiated by the findings of a monomannophosphoinositide, and an enzyme system for its biosynthesis in propionibacteria (17). However, it was later shown that the bulk of the mannose-inositol-containing lipids of propionibacteria were not glycerides and that the monomannophosphoinositide was a minor component (18,19). In view of the relationship between propionibacteria and corynebacteria (1) it was therefore possible that the mannose-inositol lipids of the latter were not mannophosphoinositides but mannoinositides, a possibility we have excluded in this work.

Some of these results have been published in preliminary form (20,21).

MATERIALS AND METHODS

Organisms

Corynebacterium diphtheriae, *Corynebacterium equi* and *Corynebacterium xerosis* were obtained from the Department of Bacteriology, Trinity College, Dublin, and were grown, harvested and washed as described previously (7). The origins of *Corynebacterium ovis* have been described by Carne et al. (22).

Lipid Extraction

In the early stages of this work the cells were first extracted several times with redistilled acetone. This treatment removed acylglucoses (7); phospholipids were then obtained by subsequent extraction of the bacterial residue with chloroform-methanol-water (16:6:1, all ratios are by volume). In later experiments cells were extracted only with chloroform-methanol-water. Lipid extracts were washed (23), dried

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by rotary evaporation and stored under nitrogen at 4 C. Prior to column chromatography phospholipid mixtures were emulsified, a small amount of sodium EDTA was added and the emulsion was stored at 4 C for two days. The preparation was then dialyzed against 0.1 M sodium EDTA for 24 hr and against deionized water for a further 24 hr. Finally the material was lyophylized and chromatographed. In the case of *C. ovis*, soluble lipids were repeatedly triturated with acetone. Acetone-insoluble lipids only were further examined.

Chromatographic Procedures

Total lipid extracts were applied to a column of silicic acid (Mallinckrodt) which was eluted first with 2-3 column volumes of chloroform, followed by varying concentrations of acetone in chloroform to remove acylglucoses and acyltrehaloses (7). Phospholipids were then eluted with chloroform-methanol (1:1).

Column chromatography of the phospholipids of *C. diphtheriae* was carried out on DEAE-cellulose (Whatman DE-32) (acetate form) in chloroform-methanol-water (20:9:1) with ammonium acetate gradients (16). Collected fractions (3 ml) were monitored for carbohydrate and phosphorus. Mixed fractions were dialyzed to remove ammonium acetate and chromatographed on thin layer plates. Such lipids were also hydrolyzed or deacylated and the products chromatographed on paper.

Deacylated phospholipids of *C. ovis* were chromatographed on a column of DEAE-Sephadex (14). The column was eluted first with water, followed by a gradient of ammonium carbonate (0.3.0 M). Ammonium carbonate was removed from eluates by repeated lyophylization and fractions were further purified by passage through a column of Sephadex G-25.

Thin layer chromatography (TLC) of phospholipids was performed on plates (20 x 20 cm and 20 x 40 cm) of Silica Gel H (Merck). The following solvent systems were used: chloroform-methanol-water (65:25:4) (solvent A); chloroform-methanol-acetic acid-water (30:15:4:2) (solvent B); chloroform-methanol-7 N ammonia (12:7:1) (solvent C). Lipids were located by exposing the plates to iodine vapor and also by gentle spraying of the plates with water. Carbohydrate-containing lipids were detected with a spray composed of 1% phenol in 60% orthophosphoric acid. Phospholipids were detected with the molybdenum-blue reagent (24). A ninhydrin spray was also used for this purpose. In preparative TLC, phospholipids were located by spraying a strip with molybdenum blue reagent or the whole plate with water, bands were scraped from the plates and the lipids eluted from the gel with chloroform-methanol (1:2) and chloroform-methanol (2:1). Eluted lipids were dried by rotary evaporation and washed (23).

Paper chromatography was performed on Whatman No. 1 or 3 MM paper, using the following solvent systems: Ethyl acetate-acetic acid-formfc acid-water (18:3:1:4) (solvent D); ethyl acetate-pyridine-water (2:1:2, upper phase) (solvent E); isopropyl alcohol-ammonia-water (7:1:2) (solvent F). Solvent D proved to be the only one which could effectively resolve glucose, mannose and arabinose. Solvent E was capable of separating mannose and arabinose. Solvent F was used for the separation of glyceryl-phosphoryl derivatives. Separated products were located by staining the papers with the silver nitrate-sodium hydroxide dip reagent (25), the periodate-benzidine dip reagent (26) or the aniline-phthalate spray (27).

Gas liquid chromatography (GLC) of methylated fatty acids was carried out on a Pye Unicam Series 104 Analyzer, using a column of 10% diethylglycolsuccinate (DEGS) on Chromosorb W, (100/120 mesh). Assignments for each peak were obtained from a semi-logarithmic plot of relative retention times against chain length and degree of unsaturation of standard mixtures of fatty acid methyl esters. The percentage of each acid was estimated from the ratio of the weight of a peak tracing to total weights of all peaks.

Analytical Methods

Carbohydrates were estimated by a phenol-H_2SO_4 assay method (28). Total phosphorus was determined by the method of Bartlett (29) and amino compounds by the method of Spies (30). Glycerol was estimated by the amount of formaldehyde produced on periodate oxidation (31). Acid hydrolysis of lipids was carried out with 2 N HCl for 3 hr at 100 C in sealed tubes. Lipids were deacylated by mild NaOH treatment. Methyl esters of fatty acids were obtained either by transmethylation or by saponification followed by methylation with diazomethane.

Materials

Phosphatidylinositol, phosphatidylethanolamine and phosphatidylserine were isolated from yeast (32,33). Dimannophosphoinositides were isolated from *Mycobacterium phlei* (16,34). Straight chain and branched chain fatty acids for GLC were obtained from Applied Science Laboratories, State College, Pa.

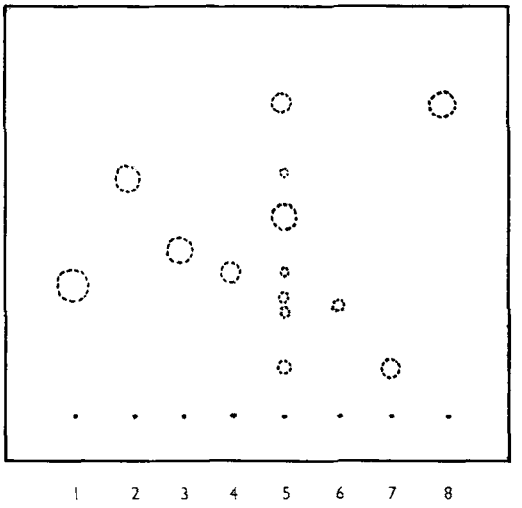

FIG. 1. Thin layer chromatogram of the total phospholipid fraction from *Corynebacterium diphtheriae*, developed with chloroform-methanol-water (65:25:4) and sprayed with the molybdenum blue reagent. 1, phosphatidylinositol; 2, phosphatidylethanolamine; 3, dimannophosphoinositide A; 4, phosphatidylserine; 5, total phospholipid from *C. diphtheriae;* 6, dimannophosphoinositide B; 7, dimannophosphoinositide C; 8, cardiolipin. (Phospholipids 2-4 were included as markers, but, as mentioned in text, they were absent from *C. diphtheriae*.)

FIG. 2. Thin layer chromatogram of the *Corynebacterium diphtheriae* phospholipid fractions eluted from the DEAE-cellulose column. PE, phosphatidylethanolamine; PS, phosphatidylserine; 1, early elutes from the column; 2, phospholipids eluted with 0.05 M ammonium acetate; 3,4,5, material eluted with higher concentrations of ammonium acetate; PI, phosphatidylinositol. Chromatography was carried out on long (40 cm) plates of Silica Gel H in chloroform-methanol-water (65:25:4). Dimannophosphoinositide C' (Table II) is not obvious on this photograph. Dotted areas contained lipids which reacted with iodine previous to visualization with the phosphate spray.

RESULTS

Phospholipids of Corynebacterium diphtheriae

Acid hydrolysis of total free lipid of *C. diphtheriae* and chromatography in solvent D showed mainly glycerol, mannose, glucose and inositol. Ethanolamine and serine were absent from chromatograms stained with ninhydrin. Chromatography of the deacylated lipid in solvent F showed glucose, trehalose, glycerylphosphorylinositol, glycerylphosphorylinositol dimannoside, and an unknown of $R_{glucose}$ 0.71. [A photograph of the products obtained has previously been published (7) in connection with the identification of acylglucoses in *C. diphtheriae*.] Glucose arose from acylglucose (7). Trehalose was probably due th the presence of diacyltrehalose (cord factor) (6). The origins of the glycerylphosphorylinositol and the glycerylphosphorylinositol dimannoside were investigated further by TLC in solvents A and B. Plates were sequentially visualized with iodine, ninhydrin and the molybdenum blue reagent. Ninhydrin positive lipids were absent as reported previously for *C. exerosis* (20), thus excluding the possible presence of phosphatidylethanolamine or phosphatidylserine in *C. diphtheriae*. The molybdenum blue reagent showed the presence of several phospholipids (Fig. 1). The major lipid (R_f [0.56 in solvent A) is considered later. Another phospholipid had a R_f of 0.42 close to that of phosphatidylinositol. Two further phospholipids chromatographed to the same region (R_f 0.35) as the triacylated dimannophosphoinositide B. Other prominent phospholipids corresponding to the diacylated dimannophosphoinositide C from *M. phlei* (16) and to cardiolipin from *M. tuberculosis*.

To purify and further identify the individual phospholipids, lyophilyzed lipid was applied to a column of DEAE-cellulose and eluted as described. Acylglucoses and the traces of

TABLE I

Characterization of Phosphoinositides Isolated From *Corynebacterium diphtheriae*[a]

R_f value of lipid in solvent A	Products of acid hydrolysis	Products of deacylation	Probable identity of lipid
0.13	Inositol, mannose, glycerol	Glycerylphosphoryl-inositol dimannoside	Diacyl dimannophos-phoinositide C
0.25	Inositol, mannose, glycerol	Glycerylphosphoryl-inositol dimannoside	Diacyl dimannophos-phoinositide C'
0.32	Inositol, mannose, glycerol	Glycerylphosphoryl-inositol dimannoside	Triacyl dimannophos-phoinositide B
0.34	Inositol, glycerol	Glycerylphosphoryl-inositol	Phosphatidyl-inositol

[a]Fractions 3 and 4 (Fig. 2) from DEAE-cellulose column were applied as bands to long (40 cm) plates of silica gel H and chromatographed in chloroform-methanol-water (65:25:4). Phospholipids were located with water and by spraying a side strip with molybdenum blue. Chromatography on these longer plates fully resolved the dimannophosphoinositides B and C' which chromatographed close together on 20 cm plates (Fig. 1).

diacyltrehaloses were removed with chloroform-methanol-water. The pattern of phospholipids eluted with ammonium acetate is shown in Figure 2. The major unknown phospholipid and a phospholipid corresponding to dimannophosphoinositide B were eluted with the gradient up to 0.05 M ammonium acetate (Fraction 2). Phospholipids corresponding to phosphatidylinositol, dimannophosphoinositide B, dimannophosphoinositide C and some unknowns were subsequently eluted (Fractions 3 and 4). The two phospholipids in Fraction 2 were isolated by preparative TLC in solvent A. The minor one on deacylation yielded glycerylphosphorylinositol dimannoside and hydrolysis showed mannose, glycerol and inositol only. Its R_f value would indicate the triacylated dimannophosphoinositide B (16). The major lipid in this fraction was the predominant single phospholipid in *C. diphtheriae*. This was repurified twice in solvent B; such purifications yielded a nitrogen-free phospholipid. Acid hydrolysis of the lipid followed by paper chromatography and visualization with the silver nitrate-sodium hydroxide reagents showed glycerol only. Assay for glycerol and phosphorus gave a molar ratio of 1.85:1. In three solvents the lipid was chromatographically similar to phosphatidylglycerol. It had the following $R_{phosphatidylethanolamine}$ values: 0.78 in solvent A, 0.90 in solvent B and 1.10 in solvent C. Paper chromatography of the deacylated lipid in solvent F showed a product with an $R_{glycerylphosphorylinositol}$ value of 1.66 compared to 1.68 for glycerylphosphorylglycerol. Positive identification of this lipid was not obtained, but the above evidence is strongly indicative of phosphatidylglycerol.

Four phospholipids were isolated from Fractions 3 and 4 by preparative TLC in solvent A. Identification of these is summarized in Table I. A number of dimannophosphoinositides are obviously present as well as phosphatidylinositol.

Phospholipids of Corynebacterium xerosis, Corynebacterium equi and Corynebacterium ovis

Brennan (20) previously examined the phospholipids of *C. xerosis* and described the major components, a phosphoinositide and a dimannophosphoinositide. In the present work the organism was re-examined for the possible presence of phosphatidylglycerol and other dimannophosphoinositides. Six phospholipids were isolated by preparative TLC in solvent A. The principal ones were identical to the dimannophosphoinositide and phosphoinositide described before (20). Another prominent phospholipid was present with chromatographic properties identical to dimannophosphoinositide C from *M. phlei* and on deacylation yielded glycerylphosphorylinositol dimannoside. Hydrolysis produced only inositol, mannose and glycerol. Two further phospholipids (R_f 0.76 and R_f 0.94 in solvent A) have not been fully identified. The former on hydrolysis yielded glycerol, glucose and inositol. The latter had chromatographic properties identical to cardiolipin.

The products of acid hydrolysis of the total soluble lipid from *C. equi* were glycerol, mannose, inositol, glucose and arabinose. The major products of deacylation were identified as glycerylphosphorylinositol dimannoside,

glycerylphosphorylinositol, glycerol and glucose. A prominent product had an $R_{glucose}$ value of 0.70, similar to the glycerylphosphorylglycerol characterized above.

Hydrolysis of the acetone insoluble lipids of *C. ovis* yielded glycerol, glucose, arabinose, mannose and inositol. A portion (1.03 g) of this material was deacylated, yielding 268 mg of water soluble products. These were fractionated on a column of DEAE-Sephadex and identified by chromatography in solvent H. The products were glycerol, arabinose, trehalose, glycerylphosphorylinositol and glycerylphosphorylinositol dimannoside. Since Lacave et al. (35) reported the presence in this organism of phospholipids containing inositol and arabinose, it was possible that we had a glycerylphosphorylinositol diarabinoside instead of the dimannoside. This material was therefore further examined. It was first purified on a column of Sephadex G-25 and rechromatographed in several systems where it had chromatographic properties identical to glycerylphosphorylinositol dimannoside. The deacylated phospholipid was hydrolyzed and chromatographed in solvents D and E for long periods to resolve arabinose and mannose. Glycerol was lost from the end of these chromatograms; however, they showed the presence of both inositol and mannose and not arabinose (Fig. 3). Moreover, staining the chromatogram with aniline-phthalate showed that the monosaccharide yielded a brown color, characteristic of hexoses and not the purple color of pentoses. Thus, this lipid which is prominent in *C. ovis*, is a dimannophosphoinositide and not a diarabinophosphoinositide.

Fatty Acids of the Phospholipids of Corynebacteria

TLC in benzene (36) of the methylated phospholipid fatty acids from *C. diphtheriae, C. xerosis* and *C. equi* showed they were non-hydroxylated, i.e., they had an R_f similar to methyl stearate. On the other hand, the methylated fatty acids from the acetone insoluble lipids of *C. ovis* were seen to contain considerable material with the chromatographic properties of methyl mycolate. This is in agreement with the finding of corynomycolic acid in a similar fraction by Lacave et al. (35) which probably arises from dicorynomycolyltrehalose (6). The methylated phospholipid fatty acids of *C. ovis* and *C. equi* as well as the fatty acids from the four dimannophosphoinositides of *C. diphtheriae* were then examined by GLC (Table II). The major fatty acid of the dimannophosphoinositides of *C. diphtheriae* closely corresponded to $C_{16:1}$; in dimannophosphoinositide B it comprised almost half of the total fatty acid content. A feature of these patterns was the prominence of branched chain fatty acid types. The percentage of these ranged from 42.3% in one sample of dimannophosphoinositide C to 26.5% in dimannophosphoinositide B of *C. diphtheriae*. The major type found in all four dimannophosphoinositides was the branched chain C_{15} fatty acid. Branched chain fatty acids also predominate in the phospholipids from the other species comprising 66.9% in *C. equi* and 91.8% in *C. ovis*.

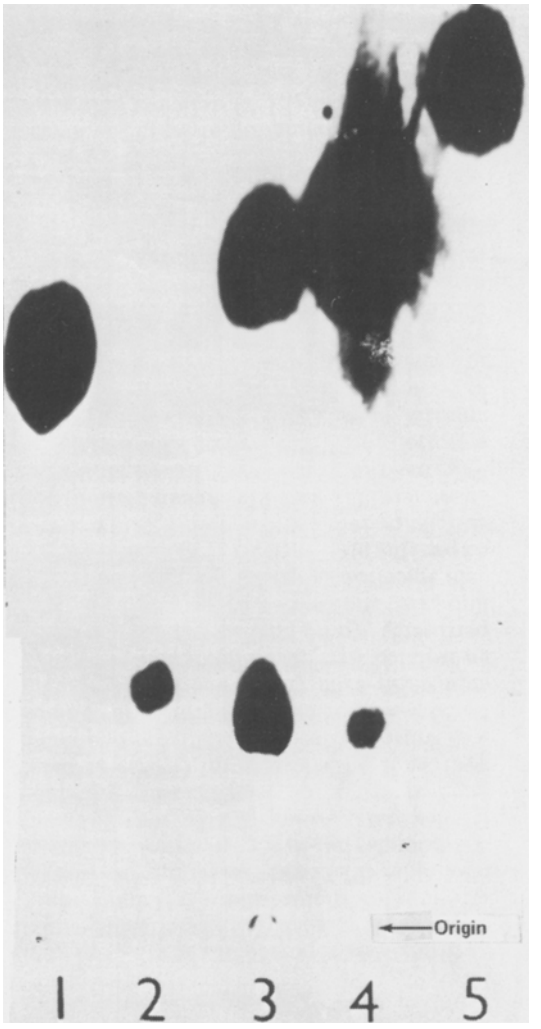

FIG. 3. Hydrolyzed products of deacylated dimannophosphoinositide from *Corynebacterium ovis* chromatographed in a descending direction in ethyl acetate-acetic acid-formic acid-water (18:3:1:4) and stained with the silver nitrate-sodium hydroxide dip reagent. Glycerol was lost from the end of this chromatogram. 1, glucose; 2, inositol; 3, hydrolyzed products; 4, mannose and inositol; 5, arabinose.

TABLE II

Fatty Acid Composition of the Dimannophosphoinositides of *Corynebacterium diphtheriae* and of the Total Phospholipid Fractions From *Corynebacterium equi* and *Corynebacterium ovis*[a]

Organism	Phospholipid	Fatty acid[c]									
		Branched 14:0[b]	14:0	Anteiso 15:0	15:0	Iso 16:0	16:0	16:1	Iso 17:0[b]	Anteiso 17:0	18:1
C. diphtheriae	Dimannophospho-inositide C[d]	9.4	10.6	16.2	17.0	11.5	10.0	13.2		6.2	5.9
	Dimannophospho-inositide C[d]	5.3	7.8	12.2	10.1	8.4	13.9	25.6		6.6	10.1
	Dimannophospho-inositide C′	5.8	8.9	14.2	12.6	10.5	10.0	30.3		7.6	
	Dimannophospho-inositide B	6.2	8.4	11.0	10.0	5.7	9.6	45.5		3.6	
C. equi	Total phospholipid		10.5	9.6	15.1		7.5		49.1	8.2	
C. ovis	Total phospholipid		1.7	1.8	1.6		7.9		55.0	32.0	

[a]Composition is expressed as percentage of total fatty acids in each lipid.

[b]Identification of these fatty acids is tentative.

[c]Number to the left of colon refers to number of carbon atoms; number to the right refers to number of double bonds.

[d]Two different preparations.

TABLE III

Hypothetical Evolutionary Descent of Mannose-Inositol-Containing Lipids

Genus	Type of lipid	Reference
Propionibacterium	Monomannoinositide and monomannophosphoinositide	18,19,17
Corynebacterium	Dimannophosphoinositides	This work
Mycobacterium	Mono-, di-, tri-, tetra-, penta- and hexamannophosphoinositides	13,14

The percentage of saturated straight chain fatty acids varied. They comprised an average of 32% in the four dimannophosphoinositides of *C. diphtheriae* and 33.1% in *C. equi*; however in *C. ovis* this figure is reduced to 11.2%. The phospholipids of *C. ovis* and *C. equi* seem to contain most of the fatty acid types present in the dimannophosphoinositides of *C. diphtheriae*, viz. C_{14}, anteiso C_{15}, C_{15}, C_{16} and branched C_{17}; they lack however, branched C_{14}, iso C_{16} and $C_{18:1}$. The most salient point of difference between the phospholipid fractions is, however, that concerning the dominant single isomer in each fraction; in *C. ovis* and *C. equi*, the $C_{16:1}$ acids of *C. diphtheriae* appear to be replaced by an iso C_{17} fatty acid.

DISCUSSION

The most striking feature of the phospholipids of the corynebacteria examined here is the predominance of phosphoinositide-type lipids, consisting of phosphatidylinositol and dimannophosphoinositides. Unlike mycobacteria, we found no evidence in corynebacteria for mannophosphoinositides other than the dimer. Chromatographic evidence indicated that these dimannophosphoinositides are identical to the triacylated and diacylated dimannophosphoinositides of *M. phlei* but no evidence was found for the tetraacylated dimannophosphoinositide prominent in *M. phlei* and *M. tuberculosis*.

The existence of dimannophosphoinositides in several corynebacteria substantiates the proposed relationship between corynebacteria and mycobacteria. This similarity has received recent fresh support from the computer analyses of corynoform bacteria of Masuo and Nakagawa (37). These authors calculated similarity indices for 12 Gram-positive genera based on phenetic characters as diverse as cell shape and size, stain reactions, cell wall components and nutritionals for growth. Their results showed an overall similarity of approximately 70-75% between corynebacteria and mycobacteria. The differences found in the composition of the mannophosphoinositides does not detract from the proposed relationship but serves rather to reinforce the existence of corynebacteria as a separate genus.

It is interesting to compare the types of mannoinositides found in the most studied genera, i.e., propionibacteria, corynebacteria and mycobacteria (Table III). Assuming an evolutionary line of descent from monomannoinositides and monomannophosphoinositides to polymannophosphoinositides, the ensuing pathway would match that of Lechevalier and Lechevalier (1). This would also be in agreement with the evolutionary pathway of mycolic acid structure in several of these genera (38). Unfortunately, little is known of the mannose-containing inositides of the nocardias, but on the basis of their mycolic acid structure they are in an intermediate position between the corynebacteria and mycobacteria.

The phosphoinositides of *C. ovis* represent a special case. Lacave et al. (35) concluded that these were principally arabinophosphoinositides. The fractionation procedure was based on a traditional method largely utilizing the differential solubility of lipids in various organic solvents and does not seem to have been carried out with the ultimate aim of identifying inositol-containing phospholipids. Using more direct procedures, we found two major deacylated phospholipids with chromatographic properties similar to those of glycerylphosphorylinositol and glycerylphosphorylinositol dimannoside. Since the latter compound would be expected to behave chromatographically in a similar fashion to a diarabinoside derivative, the monosaccharides of the compound were examined and shown to be mannose rather than arabinose. This is not to suggest that arabinophosphoinositides do not occur in *C. ovis* but that they are probably minor components, possibly on a level with the reported traces of arabinophosphoinositides in *M. tuberculosis* (38).

The major single phospholipid from *C. diphtheriae* exhibited certain unusual features. Hydrolysis of an apparently pure sample produced one ninhydrin-positive spot in a one-dimensional chromatographic system (21). However two-dimensional chromatography showed several amino acids in about equal amounts (D.P. Lehane, unpublished work). Repeated thin layer chromatography of this material finally yielded a preparation free of nitrogen and analyses indicated phosphatidylglycerol. The occurrence of phosphatidylglycerol as the major phospholipid of *C. diphtheriae* seems to represent a point of departure from phylogenetic patterns. Although phosphatidylglycerol is widespread in Gram-positive bacteria (40) it does not appear to be present in appreciable amounts, if at all, in mycobacteria or in nocardia (41); it has however, been reported among the phospholipids of *Propionibacterium shermanii* (18).

The most prominent feature of the fatty acid patterns of the corynebacterial phospholipids is the predominance of branched chain isomers. In the phospholipids of *C. diphtheriae*, the principal branched chain fatty acid corresponds to anteiso C_{15}, which is in keeping with the finding of Moss et al. (42) that the single most abundant fatty acid in *Corynebacterium acnes* is a C_{15} branched chain acid. The range of remaining fatty acids in *C. diphtheriae* is substantially similar to that observed by Moss et al. (42) in *C. acnes* though differing in relative amounts. The main point of divergence would appear to be that the most abundant single fatty acid present in the phospholipids of *C. diphtheriae* corresponds to $C_{16:1}$. The phospholipid fatty acid patterns of *C. ovis* and *C. equi* also show large quantities of branched chain fatty acids; however in these organisms the principal isomer would appear to be a branched-chain C_{17} acid. Although the fatty acids of these organisms are qualitatively similar to those of *C. diphtheriae*, they are quantitatively different. The genus *Corynebacterium* is composed of a very diverse range of organisms, many of which might be better classified as propionibacteria, or as brevibacteria (42,43). It is not surprising therefore to find different members of this genus with quantitatively distinct fatty acid patterns.

Comparison of the fatty acid patterns of the phospholipids of *C. diphtheriae* with those of the phospholipids of mycobacteria, nocardia and streptomyces (41,44) and with the total fatty acids of listeria (45) reveal overall similarities between the genera. Branched chain fatty acids are prominent, and most straight chain fatty acids from C_{14} to C_{18} are also present. This similarity is most marked between corynebacteria and streptomyces (46) and between corynebacteria and nocardia (47). Between corynebacteria and mycobacteria the major divergence in pattern is found in the fact that the branched chain C_{15} and C_{17} fatty acids of the phospholipids of the corynebacteria are replaced in the mycobacteria by the branched C_{19} fatty acid, tuberculostearic acid (48).

The biological role of the dimannophosphoinositides of corynebacteria is now under consideration in this laboratory. In mycobacteria such lipids are mainly located in the cell envelope and it has been suggested that these lipids are the primary structural components of the cell. The low turnover rate of the mannophosphoinositides supports this hypothesis (49). Shaw and Dinglinger (19) suggest that the hydrophilic portions of the mannoinositides of propionibacteria form pores in the membranes through which small ions and molecules may pass. Such a function may apply to the phosphorylated inositides.

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Quantitative Analysis of the Phospholipids of Some Marine Bioluminescent Bacteria

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ABSTRACT

Quantitative analyses of the phospholipids of three strains of marine bioluminescent bacteria were carried out after separation by two-dimensional thin layer chromatography. The phospholipids of all three species consisted of about 75% phosphatidyl ethanolamine, 13% phosphatidyl glycerol and 7% cardiolipin. The composition was only slightly affected by drastic changes in the growth conditions. One of the species contained poly-β-hydroxybutyrate. The fatty acids of another species contained principally straight and branched-chain 16 and 18 carbon fatty acids. No clue as to the nature of the elusive "aldehyde factor" of bacterial bioluminescence was found by analysis of aldehyde deficient mutants, indicating that possibly the factor is not a major phospholipid of these bacteria.

INTRODUCTION

Precise quantitative analyses of the phospholipids of bacteria, especially of marine bacteria, are as yet relatively rare (1-4). This paper presents data for the phospholipids of three strains of marine bioluminescent bacteria. The lipids of these bacteria are of more than general interest since the in vitro bioluminescence reaction requires long chain aldehydes such as dodecanal (5). The in vivo bioluminescence may also depend on aldehydes. Certain "aldehyde" mutants normally glow very dimly but, upon addition of aldehydes, transiently reach nearly wild type levels of luminescence (6,7), indicating that wild type cells contain an "aldehyde factor" which may be lacking in the mutants. No such aldehyde factor has yet been demonstrated by extraction experiments. Since the aldehyde factor may be related to phospholipid composition, several aldehyde mutants of one of the strains were analyzed.

MATERIALS AND METHODS

All chemicals were reagent grade. Solvents were distilled from glass before use. Methyl 14-methylpentadecanoate and methyl 16-methylheptadecanoate were obtained from Applied Science Laboratories, State College, Pa. 2,6-di-*tert*-butyl-*p*-cresol (BHT) was obtained from Eastman Organic Chemicals, Rochester, N.Y.

Three strains of bacteria were used: *Photobacterium fischeri*, ATCC 7744, *P. fischeri* strain MAV (MAV) (8), and *P. phosphoreum* from the collections of C.B. van Niel. Light production was measured with an apparatus similar to that described by Hastings and Weber (9) and OD measurements were made with a Beckman DU spectrophotometer with a red filter (Corning 2-61) placed between the sample and the phototube. The bacteria were grown at room temperature (ca. 25 C) under three different sets of conditions: (a) "complete, small batch," 5 g of Bacto-tryptone, 3 g of yeast extract, and 2 ml of glycerol per liter, plus salts (10), with very vigorous aeration achieved with a stirrer fitted with a propellor paddle, using 1 or 2 liters of medium; (b) "complete, large batch," as above, but with 10 or 20 liters of medium and with less vigorous aeration achieved with air bubbler tubes; and (c) "minimal," 2 ml of glycerol per liter, plus salts (10), with aeration as in (a) above. In one experiment, arginine was added at a concentration of 1.2 g/liter. Arginine is known to increase the bioluminescence of cells growing in minimal media partly by increasing the availability of the aldehyde factor (11).

Wild type MAV were grown under all three sets of conditions, *P. fischeri* only under the first two, and *P. phosphoreum* and MAV aldehyde mutants only under the first. Table I shows the OD at 660 nm and the light output of the cultures at the time of harvesting. Cells were harvested by centrifuging for 10 min at 0 C at 12,000 x *g* and were washed three times with cold 3% NaCl. At the end of the harvesting procedure, wild type cells which were resuspended in 3% NaCl and allowed to warm to room temperature were still motile and nearly as luminescent as before. Cells were frozen at -20 C immediately after harvesting.

The extraction, separation and phosphorus analysis of the lipids followed the procedures described previously (12,13). A 1 g sample of a frozen bacterial pellet was extracted with 20 ml

TABLE I

OD and Light Output of *Photobacterium* Cultures at Time of Harvesting

Strain	Growth conditions[a]	OD at 660 nm	Light output quanta $sec^{-1} ml^{-1}$	Maximum light output after addition of aldehyde[b] quanta $sec^{-1} ml^{-1}$
MAV[c]	Complete, small batch	1.6	3.6×10^{11}	---
MAV	Complete, large batch	ca. 3.[d]	ca. 1×10^{11}[d]	---
MAV	Minimal	0.85	4×10^{6}	---
MAV	Minimal + arginine 1	0.49	2.7×10^{10}	---
MAV	Minimal + arginine 2	0.95	1.0×10^{9}	---
1A mutant[e]	Complete, small batch	1.5	4.0×10^{7}	8.0×10^{10}
5A mutant	Complete, small batch	1.6	5.4×10^{8}	2.0×10^{11}
7A mutant	Complete, small batch	1.7	4.6×10^{8}	1.9×10^{11}
10A mutant	Complete, small batch	1.4	2.0×10^{8}	2.0×10^{10}
11A mutant	Complete, small batch	1.8	6.0×10^{8}	1.0×10^{11}
12A mutant	Complete, small batch	1.5	8.0×10^{6}	1.9×10^{10}
P. fischeri	Complete, small batch	1.3	1.0×10^{12}	---
P. fischeri	Complete, large batch	ca. 3.6[d]	ca. 1×10^{10}[d]	---
P. phosphoreum	Complete, small batch	ca. 2.1	not determined	---

[a]See Materials and Methods for explanation.

[b]A 0.02 ml sample of an ultrasonicated suspension of 0.02 ml of dodecanal in 10 ml of water was added to 1 ml aliquots of cultures of the mutants.

[c]MAV: *Photobacterium fischeri* strain MAV.

[d]These figures are approximate, since harvesting took place over relatively long periods of time for the large batch cultures.

[e]All mutants were aldehyde mutants of strain MAV.

of chloroform-methanol 2:1 (containing 50 μg of BHT), 10 ml of chloroform-methanol 2:1, 10 ml of chloroform-methanol 1:2 and 10 ml of chloroform-methanol 7:1 saturated with 28% aqueous ammonia (13). The combined extracts were evaporated nearly to dryness with a rotary evaporator and the residue was treated several times with chloroform to remove water by replacement (12). The moist residue was suspended in a small volume of chloroform-methanol 19:1 saturated with water, applied to a 1x10 cm column of Sephadex G-25 and eluted with 30 ml of the same solvent (13). The eluate was evaporated as above and diluted to the desired volume with chloroform-methanol 9:1. The total weight of lipid in the final solution was determined by evaporating a small aliquot at 80 C for 3 min and weighing the residue with a Cahn electrobalance (12). The extracts were stored under nitrogen at -20 C.

Extracts of *P. phosphoreum* prepared according to this procedure were extremely viscous. Addition of four volumes of methanol gave a precipitate which was collected by centrifugation. The precipitate was dissolved in chloroform and reprecipitated by the addition of 4 vol of methanol four more times, and was then dried in air. An IR spectrum of this material was identical to that of authentic poly-β-hydroxybutyrate (14). The combined supernatants, containing the lipids, were evaporated and taken up in chloroform-methanol 9:1 as above. Neither MAV nor *P. fischeri* contained any of the polymer, whereas it accounted for about 4% of the wet weight of *P. phosphoreum*.

Aliquots of the lipid extracts were spotted at 55% relative humidity under nitrogen onto thin layer chromatography (TLC) plates (Silica Gel H, plain, Merck) and developed in the first dimension in chloroform-methanol-28% aqueous ammonia 13:17:1. After drying for 10 min under N_2, the plates were run in the second dimension in chloroform-acetone-methanol-acetic acid-water 6:8:2:2:1 (12). The chromatograms were dried briefly in air. For determination of total phosphorus in the extracts, aliquots of the extracts were spotted onto areas of the plates known not to contain phospholipids. The plates were then sprayed with 98% H_2SO_4-37% HCHO 97:3, charred by heating at 180 C for 30 min, and the phosphorus content of the aspirated TLC spots measured (15).

Gas liquid chromatography (GLC) was carried out with an F&M model 810 apparatus, using 4% SE-30 on 60-80 mesh Diatoport S, 1/4 in. x 6 ft, and a 10 C/min temperature rise starting at 100 C. IR spectra were taken with a Beckman model IR-4, using a micro KBr disc technique (16). NMR spectra were taken with a Varian T-60 apparatus.

RESULTS

Three major phospholipids were found in all extracts: phosphatidyl ethanolamine (PE), phosphatidyl glycerol (PG) and diphosphatidyl glycerol or cardiolipin (DPG). They were identi-

TABLE II

Phospholipid Composition of *Photobacterium* Strains as Per Cent of Total Phosphorus[c]

Strain and growth conditions[a]	Phosphorus %[b]	PE[d]	PG	DPG	LPE	origin	front	1	2	3	4	5	Other
MAV-1	3.64	75.2	12.7	5.0	2.1	0.6	NA	0.4	0.8	---	1.8	---	1.5
	3.62	76.3	12.8	4.9	2.4	0.5	(3.7)	0.2	0.9	---	2.0	---	NA
MAV-1	3.56	74.9	12.8	6.3	3.1	0.2	NA	0.1	0.8	---	0.9	---	0.8
MAV-2	3.54	74.7	13.5	6.0	2.5	0.7	(5.2)	0.3	0.3	---	1.2	0.7	---
MAV-2	3.13	72.0	15.3	8.8	2.3	0.4	(5.0)	0.2	0.6	---	---	NA	0.4
MAV-2	3.41	73.7	13.8	7.5	3.4	0.3	NA	0.2	0.4	---	---	0.5	0.3
MAV-3a	3.67	78.7	12.2	6.5	1.4	0.3	NA	0.1	0.5	0.2	---	---	---
	3.49	78.6	12.3	7.0	1.5	0.2	(2.2)	0.0	0.4	---	---	---	---
MAV-3b	3.73	76.0	13.9	7.5	1.2	0.3	NA	0.1	0.5	0.3	0.2	---	---
	3.53	75.7	14.4	8.1	1.2	0.1	(1.9)	0.0	0.4	---	0.1	---	---
MAV-3c	3.77	81.2	8.6	6.8	1.2	0.3	NA	0.0	1.0	0.3	0.7	---	---
	3.54	81.1	9.2	6.9	1.6	0.3	(1.3)	0.0	1.2	---	0.4	0.1	---
1A-1	3.41	71.8	12.1	7.2	5.7	0.1	NA	0.3	1.1	---	0.7	---	1.0
5A-1	3.33	76.7	14.7	6.2	1.1	0.1	NA	0.1	0.7	0.3	---	---	---
7A-1	3.40	73.6	16.1	6.5	1.5	0.0	(3.4)	0.3	0.7	0.2	0.2	---	0.8
10A-1	3.35	73.6	15.9	7.8	1.8	0.1	(0.8)	0.2	0.3	0.3	---	---	---
	3.42	74.1	15.6	7.6	1.8	0.1	(0.7)	0.2	0.3	0.4	---	---	---
	3.62	75.2	14.2	7.6	1.7	0.0	(0.3)	0.1	0.4	0.8	---	---	---
	3.64	74.8	15.0	7.6	1.8	0.1	(0.5)	0.2	0.2	0.3	---	---	---
11A-1	3.42	75.4	16.1	5.6	1.3	0.4	NA	0.3	0.6	0.5	---	---	---
12A-1	3.45	76.4	15.0	6.3	1.2	0.1	NA	0.2	0.5	0.3	0.2	---	---
P. fischeri-1	3.48	79.0	11.3	5.0	2.7	0.3	NA	0.2	1.1	0.4	---	---	---
	3.58	78.0	13.3	5.1	2.6	0.1	NA	0.3	NA	0.3	0.4	---	---
P. fischeri-2	3.29	74.3	7.2	7.2	1.7	0.2	NA	0.2	2.9	---	4.2	1.7	0.4
	3.32	77.0	6.5	6.6	1.8	0.2	NA	0.1	2.5	---	3.9	1.5	---
P. phosphoreum-1[e]	0.6[f]	66.3	12.8	9.2	4.6	2.3	NA	---	3.7	---	---	---	---
P. phosphoreum-1[g]	4.6[f]	73.1	13.7	6.2	5.5	0.2	(8.2)	0.2	0.5	---	---	---	0.6
P. phosphoreum-1[g]	3.0[f]	71.9	14.6	6.3	5.7	0.1	(0.2)	0.2	0.5	---	---	---	0.8
Average[h]	3.49	75.3	13.1	6.8	2.4	0.3		0.2	0.9	0.2	0.6	0.2	0.3

[a]MAV aldehyde mutants are listed as 1A, 5A, etc. Growth condition 1: complete, small batch; condition 2: complete, large batch; condition 3a: minimal; condition 3b: minimal plus arginine with the culture grown to an OD of 0.49; condition 3c: as for 3b, but growth continued to an OD of 0.95. See Materials and Methods and Table I. All entries represent separate extractions of cells. Repeat analyses of the same extracts are indicated by blanks in the first column.

[b]Grams of phosphorus per 100 g of lipid.

[c]Neglecting material at the solvent fronts (values in parentheses). See Figure 1 for identification of spots. A dash means no spot was visible. NA means not analyzed.

[d]Abbreviations: PE, phosphatidyl ethanolamine; PG, phosphatidyl glycerol; DPG, diphosphatidyl glycerol; LPE, lysophosphatidyl ethanolamine.

[e]Poly-β-hydroxybutyrate was not removed prior to analysis and was included as lipid in the calculation for column 2.

[f]Approximate values.

[g]Poiy-β-hydroxybutyrate was removed prior to analysis.

[h]Excluding approximate values. Values marked with a dash were counted as 0.0%.

TABLE III

Phospholipid Composition of *Photobacterium* Strains as Per Cent of Total Lipid Phosphorus[a]

Strain[b]	PE[c]	PG	DPG	LPE	Spot 2[d]
MAV (15)[e]	75 ± 1.5	14 ± 1.4	7 ± 1	2 ± 1	0.6 ± 0.3
P. fischeri (4)	77 ± 3	10 ± 3	6 ± 1	2 ± 1	2 ± 1
P. phosphoreum (3)	70 ± 3	14 ± 1	7 ± 2	5 ± 1	ca. 1

[a]Average values and standard deviations derived from Table II for cells grown in complete media.

[b]Numbers in parentheses indicate the number of analyses from which the averages were derived.

[c]For abbreviations see Table II.

[d]Spot 2 is tentatively identified as phosphatidic acid.

[e]Data for the aldehyde mutants are included.

fied by their migration patterns in this and several other TLC systems and by comparison of the IR spectra of material eluted from TLC spots with those of authentic materials (16-18). The phospholipids of *P. phosphoreum* migrated slightly differently from those of MAV or *P. fischeri*, indicating that the fatty acids in *P. phosphoreum* are probably different from those in the other two strains.

One sample of PE, obtained from MAV cells grown under the "complete, large batch" conditions, was further characterized. The PE was eluted from a spot on a TLC plate, passed through a Sephadex G-25 column to remove silica gel, and hydrolyzed in 6 M HCl at 110 C for 18 hr. The hydrolysate was extracted with hexane and the HCl solution was evaporated to dryness. The residue was analyzed with a Beckman model 116 amino acid analyzer. One mole of ethanolamine was found per mole of phosphorus in the spot. The hexane extract was evaporated to dryness, treated with concentrated HCl-methanol 3:8 at 110 C for 90 min (19) and extracted with hexane after the addition of water. The hexane extract, containing fatty acid methyl esters, was analyzed qualitatively by GLC. The methyl esters of 14-methylpentadecanoic acid, hexadecanoic acid, 16-methylheptadecanoic acid and octadecanoic acid were identified by a comparison of the retention times with those of authentic materials. A comparison of the IR spectrum of the ester mixture obtained by evaporation of the hexane extract with that of a mixture made from the authentic methyl-branched esters as well as an NMR spectrum of the ester mixture, confirmed the identifications (20,21). Trace amounts of other esters were also present in the hexane extract, but no unsaturated or cyclopropane fatty acid methyl esters were found.

The results of the quantitative phosphate analyses (Table II) are given as per cent of the total phosphate recovered from each plate. All spots were analyzed (Fig. 1). The total phosphate recovered, neglecting material at the solvent fronts, was about 92% of the total applied. The lipid in the extracts represents 1.0 ± 0.2% of the wet weight of the cells. Diethylaminoethyl-cellulose column chromatography (13) showed that about 6% of the lipids extracted from MAV consists of "neutral" lipids, about 2% consists of free fatty acids and the remainder consists of phospholipids. No phosphatidyl serine nor any glycolipids were found in the MAV extracts.

DISCUSSION

The results indicate that the three strains of

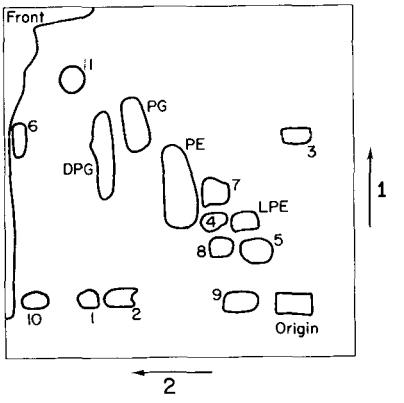

FIG. 1. Composite two-dimensional chromatogram of lipid extracts of *Photobacterium* strains. Identification of spots: PE, phosphatidyl ethanolamine; LPE, lysophosphatidyl ethanolamine; PG, phosphatidyl glycerol; DPG, diphosphatidyl glycerol; Spot 6, free fatty acid; Spot 2, phosphatidic acid. Spot 7 was seen in MAV-1 and 1A extracts, Spot 9, in *P. fischeri*-2 and *P. phosphoreum*-1 (polymer removed) extracts, the latter also showing Spot 11. Spot 10 was seen in *P. phosphoreum*-1 (polymer present) extracts. See Table II, where Spots 7-11 are listed under "Other." Chromatographic solvent systems are described in Materials and Methods.

marine bioluminescent bacteria studied are very similar in phospholipid composition. Table III shows that the approximate composition in terms of per cent of total lipid phosphorus is 75% PE, 13% PG, 7% DPG and 2% lysophosphatidyl ethanolamine. Minor phospholipids amount to about 2% of the total phospholipids. This composition is very similar to that of many other species of Gram negative bacteria (1-4,22,23).

The finding of branched chain and straight chain fatty acids and no unsaturated or cyclopropane acids in the PE of strain MAV (a Gram negative organism) is surprising since such a composition seems to be characteristic of Gram positive bacteria (3,24).

The composition of the major phospholipids of these bacteria seems to be rather insensitive to large changes in the composition of the medium. The composition of MAV grown in a minimal medium was similar to that of MAV grown in a complete medium, though growth in the presence of arginine to an O D of 0.95 (Table II, MAV-3c) seemed to raise the percentage of PE at the expense of PG. A significant change was seen with *P. fischeri* in which the amount of PG decreased while that of some of the minor components increased in high cell density cultures with the "complete, large batch" conditions (Table II) (3). There were no striking correlations between the presence or absence of any of the minor components and the nature of the strains or the conditions under which they were grown.

The composition of the major phospholipids of several aldehyde mutants of MAV is the same as that of wild type MAV. This demonstrates that the aldehyde factor of bacterial bioluminescence is not related to the amounts of the major phospholipids of these bacteria. An investigation of the minor lipids is under way. In the context of the present paper, the analyses of the aldehyde mutants serve to show the degree of reproducibility of the analytical method.

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Effects of Level and Type of Dietary Fat on Incidence of Mammary Tumors Induced in Female Sprague-Dawley Rats by 7,12-Dimethylbenz(α)Anthracene[1]

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ABSTRACT

Female Sprague-Dawley rats on semisynthetic diets containing 10% and 20% by weight of corn oil developed more mammary adenocarcinomas after treatment with a single oral dose of 7,12-dimethylbenz(α)anthracene than similar rats on diets containing only 0.5% or 5% corn oil. Experiments with 10 different fats and oils fed at the 20% level indicated that unsaturated fats enhance the yield of adenocarcinomas more than saturated fats. Fibroadenomas and adenomas were also found in small numbers in all dietary groups but the yield did not seem to be influenced by level or type of dietary fat. The possible relevance of these findings to the incidence of breast cancer in humans is discussed.

INTRODUCTION

Experiments carried out in a number of different laboratories over the past 30 years have provided evidence that rats and mice on high fat diets are more prone to develop mammary tumors than control animals on low fat diets (1,2). This applies both to spontaneous tumors (3,4) and to tumors induced by various means (5-7), and the effect seems not to be dependent on differences in caloric intake (3,7). The possible involvement of dietary fat in the etiology of human breast cancer has also received increasing attention in recent years (2,8-10).

Our studies on the effects of dietary fat have all been carried out with mammary tumors induced in female Sprague-Dawley rats by a single oral dose of dimethylbenz(α)anthracene (DMBA) (11). The initial experiments showed that rats on a high fat semisynthetic diet containing 20% corn oil developed more tumors than rats on a corresponding diet containing 20% coconut oil or on a low fat semisynthetic diet containing only 0.5% corn oil (7). In subsequent studies the level of carcinogen in mammary tissue was measured at different time intervals after injection, and although the average level was somewhat higher on the 20% corn oil diet during the first 12 hr, it seemed doubtful that the difference was sufficient to account for the higher tumor yield (12). Furthermore, it was found that the high corn oil diet enhanced the tumor yield when it was fed only after administration of the carcinogen but not when it was fed only before (13). This suggested that the effect was related to development of the tumors rather than to distribution and metabolism of the carcinogen or other factors concerned with tumor initiation.

The aim of the present experiments was to determine the effect of intermediate levels of corn oil on mammary tumor incidence and to assess the effects of a number of other dietary fats and oils.

MATERIALS AND METHODS

As in earlier studies, the experiments were carried out with 21- to 22-day-old weanling female rats obtained from Sprague-Dawley, Inc., Madison, Wisconsin. They were placed on the semisynthetic test diets as soon as received and were housed two to a cage in a temperature-controlled, well-ventilated room with artificial lighting controlled to give alternating 12 hr periods of light and darkness. The proportions of major ingredients in the diets are shown in Table I. The composition of the vitamin supplements and the sources of dietary materials have been reported previously (13). The diets were normally prepared in 2 kg lots and were stored in a cold room at 4 C.

[1]Presented at the ISF-AOCS World Congress, Chicago, September 1970.

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TABLE I

Composition of Semisynthetic Diets[a]

Diet	0.5% Fat	5% Fat	10% Fat	20% Fat
Casein	18	19	20.5	23
Dextrose	72	66.5	59.5	46
Fat	0.5	5	10	20
Salt mixture, (Phillips-Hart)	4	4	4.5	5
Celluflour	5	5	5	5

[a]Adequate vitamin supplements were added to these diets (13).

The corn oil, butter and lard used for the experiments were purchased locally. Other fats and oils were donated by Procter and Gamble, Ltd., Hamilton, Ontario. The fatty acid composition of the different dietary fats is shown in Table II. For these analyses, the fats were transmethylated by refluxing for 2 hr with a mixture prepared by adding 1 vol of acetyl chloride dropwise to 9 vol of reagent grade methanol (19). The methyl esters were then extracted with petroleum ether and analyzed by gas liquid chromatography in a Beckman GC-45 with hydrogen flame detector, using a column of 15% EGSS-X on Chromosorb P. The results were quantitated by integration and were checked by use of National Institutes of Health standard mixtures of fatty acid methyl esters.

At 50 days of age, all rats were given a single dose of 5 mg DMBA in 0.25 ml of sesame oil by stomach tube. As in previous experiments, the rats were transferred to commercial diet for two days before and one day after administration of DMBA to minimize effects of the experimental diets on absorption of the carcinogen. The DMBA was obtained from Eastman Organic Chemicals, Rochester, N.Y., and was purified by recrystallization from methanol-water as described previously (13).

The diets were continued for four months after administration of the DMBA and the rats were palpated regularly for mammary tumors. At the end of this time they were killed with chloroform and the tumors were preserved in 10% buffered formalin for sectioning and staining with hematoxylin and eosin. To minimize variations due to the use of different lots of animals at different times, the rats were allotted randomly in groups of 10 and the different experimental diets were all fed at the same time. The complete set of experiments was then repeated twice to bring the total to 30 animals per group. The only exceptions were the diets containing 10% corn oil and 20% lard. These were not included in the first feeding trial, and 20 rats were therefore allotted to each of these diets in a subsequent experiment.

The apparent digestibility of the different dietary fats was determined on groups of six rats during the course of one of the dietary experiments. The animals had been on diet about three months when these experiments were carried out. Feces were collected daily for five consecutive days and pooled for each group on a daily basis. For extraction of fecal lipids (20), 2 g of dry feces were ground in a mortar and transferred to 100 ml centrifuge tubes with 10 ml of 96% ethanol. To the mixture was added four to six drops of concentrated HCl and 2 ml of water and the lipids were extracted

TABLE II

Fatty Acid Composition of Fats and Oils (wt %)[a]

Fatty acids[b]	Shorthand designation	Coconut oil	Butter	Tallow	Lard	Olive oil	Rapeseed oil	Cottonseed oil	Corn oil	Soybean oil	Sunflower-seed oil
Caprylic	8:0	5.8	—	—	—	—	—	—	—	—	—
Capric	10:0	6.6	1.0	—	0.1	—	—	—	—	—	—
Lauric	12:0	53.5	2.5	—	0.3	—	—	—	—	—	—
Myristic	14:0	18.4	10.5	3.4	1.5	—	—	0.6	—	—	—
Palmitic	16:0	7.9	31.0	25.5	25.3	11.3	3.0	19.6	10.1	10.4	5.5
Palmitoleic	16:1	—	2.5	2.8	2.5	0.5	—	0.3	—	—	—
Stearic	18:0	1.6	13.6	24.9	15.0	2.2	1.3	2.2	1.6	3.8	4.6
Oleic	18:1	5.0	29.9	35.7	44.5	78.5	23.3	18.2	31.4	24.2	14.7
Linoleic	18:2	0.8	1.8	1.6	9.3	7.2	16.0	59.2	56.3	53.5	75.1
Linolenic	18:3	—	0.6	—	0.1	—	8.0	—	0.4	7.8	—
Eicosenoic	20:1	—	—	1.5	0.4	—	12.9	—	—	—	—
Erucic	22:1	—	—	—	—	—	34.5	—	—	—	—
	References	14	14	14	14,15	15	16	14	17	14	18

[a]Most of the analyses gave values similar to those reported in the literature (references given in Table). Butter normally contains about 4% of 4:0, and 1-2% each of 6:0 and 8:0, but these short chain acids were not determined under the conditions used for our analysis. Other minor peaks in butter and tallow were included in the calculations but are not shown in the Table.

[b]Per cent of total fatty acids.

TABLE III

Mammary Tumor Incidence Following Administration of DMBA to Rats on Semisynthetic Diets Containing Different Levels of Corn Oil

Corn oil diets	Rat body weight[a]		Tumor incidence[b]		No. of Tumors[a] per rat		No. of Tumors[a] per tumor-bearing rat	Latent[a] period, days
	Initial, g	Final, g	Total	Palpable	Total	Palpable		
20%	41.2±0.9	246.2±4.5	90.0	86.6	3.7±0.58	2.5±0.43	4.0±0.62	68.2±4.3
10%	38.8±0.3	239.9±6.6	93.3	93.3	4.0±0.48	3.0±0.41	4.3±0.46	68.0±4.5
5%	41.5±1.1	238.9±4.2	76.6	73.3	2.3±0.39	1.7±0.29	3.0±0.40	77.4±4.4
0.5%	42.9±0.5	238.6±2.5	70.0	70.0	2.5±0.40	1.6±0.35	3.5±0.40	78.4±4.5

[a]Mean ± S.E.M. Each group consisted of 30 rats.
[b]Per cent of rats with tumors at autopsy.

with two portions of 40 ml petroleum ether (30-60 C). The combined extract was transferred to a weighed beaker and dried, first under nitrogen and then overnight in a desiccator over P_2O_5. The Coefficient of Apparent Digestability was calculated as

$$\frac{\text{fat ingested - fat excreted}}{\text{fat ingested}} \times 100.$$

RESULTS

Effect of Different Levels of Dietary Fat on Mammary Tumor Incidence

The cumulative palpable mammary tumor incidence in female rats fed diets containing different levels of corn oil is shown in Figure 1 and the results at autopsy are summarized in Table III. The groups on diets containing 10% or 20% by weight of corn oil gave similar results and the tumor incidence was higher than with diets containing 0.5% or 5% corn oil. The difference between the groups on 10% corn oil and 0.5% corn oil was significant ($P<0.05$) and the group on 10% corn oil had significantly more tumors per rat than either of the low fat groups ($P<0.05$). Most of the tumors were adenocarcinomas and the differing incidence on high and low fat diets was seen only in this type of tumor (Table IV).

Effect of Different Types of Dietary Fat on Mammary Tumor Incidence

Table V shows the effect of different dietary fats, fed at a level of 20% by weight of the diet, on incidence of mammary tumors induced by DMBA. More than 85% of the animals developed tumors in all groups except those on

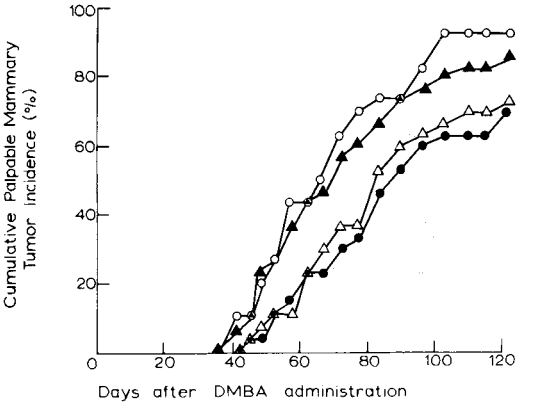

FIG. 1. Effect of diets containing different levels of corn oil on the cumulative palpable mammary tumor incidence in rats treated with DMBA. ▲——▲ 20% corn oil diet; ○——○ 10%; △——△ 5%; ●——● 0.5%.

TABLE IV

Incidence of Different Types of Mammary Tumors in Rats on Semisynthetic Diets Containing Different Levels of Corn Oil

Corn oil diets	Adenocarcinomas			Fibroadenomas			Adenomas		
	Palpable	Nonpalpable	Total	Palpable	Nonpalpable	Total	Palpable	Nonpalpable	Total
20%	76	29	105	1	2	3	2	0	2
10%	85	25	110	4	3	7	0	5	5
5%	49	17	66	0	1	1	2	1	3
0.5%	45	21	66	4	2	6	3	0	3

tallow and rapeseed oil. However, there tended to be more tumors per rat when unsaturated fats were fed and this is reflected in the trend toward higher tumor yields with increasing unsaturation in the dietary fat (Table VI). Rapeseed oil is the most obvious exception, and the low yield with this oil may be related to its high content of monounsaturated C_{20} and C_{22} fatty acids (Table II). As before, most of the tumors were adenocarcinomas and the higher yield on diets containing unsaturated fats was confined to this type of tumor (Table VI).

DISCUSSION

The results of these experiments confirm our previous findings (7,13) that young female Sprague-Dawley rats treated with DMBA develop more mammary carcinomas on a semisynthetic diet containing 20% corn oil than on a comparable diet containing only 0.5% corn oil. The tumor yield was not increased appreciably by raising the level of corn oil from 0.5% to 5%, but a further increase to 10% gave a tumor yield similar to that obtained with the 20% corn oil diet (Fig. 1, Tables III and IV). These findings are in general agreement with the results of Silverstone and Tannenbaum (3), who investigated the effect of diets containing different levels of partially hydrogenated cottonseed-soybean oil on the incidence of spontaneous mammary tumors in mice. They found that the tumor incidence increased as the level of fat in the diet was raised from 2-4% up to 12-16%, but beyond this level additional fat seemed to have little effect. Both in our experiments and in those of Silverstone and Tannenbaum, about 90% of the animals developed tumors when the diet contained 10-12% fat and it might be argued that there is not much room for further increase at higher levels. A smaller dose of DMBA could have been used in our studies to give a lower tumor incidence, but the 5 mg dose was chosen because it appeared to give the best differential in tumor yield between rats on high and low fat diets (13). It may also be noted that the number of tumors per rat showed a significant increase as the level of corn oil was raised from 5% to 10%, but no further increase in going from 10% to 20% (Table III).

Our initial studies on dietary fat in relation to incidence of DMBA-induced mammary cancer in rats indicated that coconut oil had much less effect on tumor yield than corn oil (7). Other workers have shown that edible oils such as olive oil (4), Crisco (5), and lard (6) can enhance mammary carcinogenesis in the rat, but no systematic studies have been carried out with different dietary fats. Our investigation was therefore expanded to include a variety of other edible fats and oils. The results indicated that, in general, rats on diets containing unsaturated fats developed more adenocarcinomas after treatment with DMBA than rats on similar diets containing saturated fats (Table VI). In fact, the tumor yields with dietary fats such as coconut oil and tallow were much the same as those obtained with low fat diet. (Table IV). However, although the total yield was lower with saturated fats, in most cases the percentage of animals developing tumors was about the same as for unsaturated fats. The observed differences were thus due mainly to differences in the number of tumors per rat (Table V).

Rapeseed oil was exceptional in giving a low tumor yield although it contains a relatively high proportion of unsaturated fatty acids. Much of the unsaturation, however, is accounted for by the C_{20} and C_{22} monoenes, eicosenoic acid and erucic acid (Table II). Rapeseed oil has a lower coefficient of digestibility than most other dietary fats and oils (21) and is known to depress growth in rats (22) and to increase the concentration of cholesterol in adrenals and ovaries (23). Possibly the low tumor yield is related to some of these effects.

In the present experiments, the animals in the rapeseed oil group had the lowest average weight at autopsy although it was not much below that of other dietary groups (Table V). The apparent digestibility was also lower than for any other fat except tallow (Table V), but not as low as the value reported in the literature

TABLE V

Mammary Tumor Incidence Following Administration of DMBA to Rats on Diets Containing Different Fats or Oils

Diet	Coeff. of apparent digestibility, %	Rat body weight[a]		Tumor incidence[b]		No. of tumors per rat[a]		No. of tumors[a] per tumor-bearing rat	Latent[a] period, days
		Initial, g	Final, g	Total	Palpable	Total	Palpable		
20% Coconut oil	96.8	42.1±0.9	247.1±3.1	96.6	86.6	2.4±0.33	1.8±0.29	2.5±0.38	68.8±4.6
20% Butter	94.2	41.6±0.7	252.2±2.7	86.6	83.3	2.9±0.40	2.0±0.35	3.3±0.41	73.8±3.8
20% Tallow	87.6	40.4±0.8	239.5±3.7	80.0	70.0	2.4±0.45	1.8±0.34	3.0±0.49	74.6±8.5
20% Lard	93.9	39.1±0.6	243.3±5.3	93.3	86.6	3.2±0.46	2.7±0.44	3.4±0.47	78.8±6.0
20% Olive oil	98.1	42.1±0.8	241.0±2.6	86.6	80.0	3.9±0.46	3.0±0.51	4.5±0.42	64.1±4.5
20% Rapeseed oil	92.5	42.5±0.6	237.5±5.1	76.6	73.3	2.2±0.44	1.5±0.29	2.0±0.65	71.5±4.7
20% Cottonseed oil	96.6	42.9±0.7	258.2±2.7	93.3	93.3	4.2±0.71	3.0±0.55	4.5±0.75	68.3±5.0
20% Corn oil	97.4	41.2±0.9	246.2±4.5	90.0	86.6	3.7±0.58	2.5±0.43	4.0±0.62	68.2±4.3
20% Soybean oil	97.8	42.6±0.6	252.5±1.1	100.0	86.6	3.4±0.45	2.5±0.39	3.4±0.46	70.2±4.1
20% Sunflowerseed oil	96.9	41.5±0.8	248.4±4.8	96.6	86.6	4.3±0.46	2.9±0.38	4.8±0.31	66.0±4.3

[a]Mean ± S.E.M. Thirty rats per group.
[b]Per cent of rats with tumors at autopsy.

TABLE VI

Incidence of Different Types of Mammary Tumors Following Administration of DMBA to Rats on Diets Containing Different Fats and Oils

Diets	Adenocarcinomas			Fibroadenomas			Adenomas		
	Palpable	Nonpalpable	Total	Palpable	Nonpalpable	Total	Palpable	Nonpalpable	Total
20% Coconut oil	51	18	69	2	0	2	2	0	2
20% Butter	59	20	79	1	5	6	0	3	3
20% Tallow	53	17	70	2	0	2	0	0	0
20% Lard	78	13	91	3	2	5	1	0	1
20% Olive oil	85	24	109	3	0	3	2	3	5
20% Rapeseed oil	41	21	62	3	0	3	3	1	4
20% Cottonseed oil	87	35	122	1	1	2	2	1	3
20% Corn oil	76	29	105	1	2	3	2	0	2
20% Soybean oil	76	25	101	1	0	1	0	1	1
20% Sunflowerseed oil	86	38	124	2	1	3	0	3	3

(24). Erucic acid appears to be responsible for most of the observed effects of rapeseed oil in animals (22,25) and the relatively small effects on growth and digestibility in the present experiments may be due to the use of an oil containing a lower percentage of erucic acid (16) or to feeding the oil at a lower level than those used in earlier studies.

Lard appeared to be more effective than other solid fats tested and olive oil, which contains oleic acid as the major unsaturated fatty acid (Table II), gave a tumor yield comparable to that of oils with a high content of linoleic acid (Table VI). The report of Harman (26) that increasing the level of α-tocopherol in a diet containing 20% corn oil decreased the tumor yield in rats treated with DMBA suggests that lipoperoxidation may be a factor. A recent study by Poling et al. (27) indicated that the incidence of spontaneous mammary tumors in rats fed heat-treated fats was much the same as in rats fed the untreated fats, but the peroxide values of the fats were little changed by the heating procedure. Our own studies have indicated that the effect is probably related to development rather than initiation of the tumors and it seems possible that the distribution and metabolism of steroid hormones may be altered by the level and type of fat in the diet. Further experiments are being carried out to investigate this possibility.

Examination of statistical data for human populations of different countries has disclosed a strong positive correlation between dietary fat intake and age-adjusted mortality from breast cancer (2,8,9). The findings in experimental animals may not be applicable to humans, but considered in the light of this statistical evidence, they suggest the possibility that the incidence of breast cancer might be lowered by decreasing fat intake. The high fat diets used in our studies contain about the same level of fat (20% by weight = approximately 40% of total calories) as typical American diets, whereas a low-fat diet which was effective in decreasing the incidence of mammary tumors in rats is comparable in fat content (5% by weight = approximately 10% by calories) to diets in countries such as Japan where the death rate from breast cancer is much lower than in America. The fact that unsaturated fats appeared to enhance the yield of mammary tumors to a greater extent then saturated fats in the experiments with rats also suggests that caution should be exercised in recommending a large-scale shift to more highly unsaturated fats in human diets.

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The Infrared Spectra and Polymorphism of Long Chain Esters: IV. Some Esters From Tetradecanol, Hexadecanol, Octadecanol, Eicosanol, Docosanol and Dodecanoic, Tetradecanoic, Hexadecanoic, Octadecanoic and Eicosanoic Acid[1]

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ABSTRACT

Infrared spectra and x-ray diffraction photographs have been recorded for some normal-chain esters of long chain acids and long chain alcohols. The substances show no polymorphism but two different structure types exist, one with the chains tilting towards the end group planes and one with vertical chains. The IR spectra permit determination of the chain length of the acid methylene chain. The length of the alcohol chain cannot be deduced.

INTRODUCTION

As a continuation of a program of studying fatty acid esters some wax esters have been investigated. In the earlier parts of this series (1-3) some groups of long chain esters were found to be polymorphic. It was also found that the IR spectra differed considerably between esters with long acid part and long alcohol part. A review of the field and details of the experimental technique are found in Reference 4.

Some x-ray studies on wax esters are reported in the literature. Kreger and Schamhart (5) prepared some of these esters and determined their melting points and long-spacings. By comparing the latter with calculated values they decided that, among others, octadecyl hexadecanoate crystallized in a form with vertical chains, i.e., perpendicular to the end group planes, while most others crystallized in a tilted form. Tetradecyl octadecanoate and docosyl octadecanoate gave spacings for both types of form. It was concluded that the appearance of the vertical form was a feature inherent in some of the esters and not caused by impurities.

Lutz et al. (6) accurately determined the long-spacings of several wax esters. Crystals of tetradecyl tetradecanoate, tetradecyl hexadecanoate, hexadecyl hexadecanoate, octadecyl tetradecanoate, hexadecyl octadecanoate and octadecyl octadecanoate were considered to be isomorphous and also isomorphous with crystals of ethyl esters of long acids. The powder patterns could be indexed on the basis of the unit cell of hexadecyl hexadecanoate as given by Kohlhaas (7). Hexadecyl tetradecanoate, tetradecyl octadecanoate and octadecyl hexadecanoate were found to have another form with the chain axis almost vertical. Esters of some alcohols with an odd number of carbon atoms gave several sets of long-spacing lines indicating the existence of polymorphs.

A number of studies of the dieletric absorption of the wax esters have been reported (8-12).

Iyengar and Schlenk (13) have prepared a number of the wax esters and determined their melting points. When these were plotted against the difference in the number of carbon atoms in the alcohol and the acid chains as in Figure 1, there was a striking deviation from regularity for the difference +2 in that the melting points were higher than interpolation would predict. This group of compounds includes some of those which had been claimed to have vertical chains by the earlier workers. It was considered worth while by the present authors to extend the study of the available wax esters.

EXPERIMENTAL PROCEDURES

The substances synthesized by Iyengar and Schlenck (13) have a purity of 99% or better as shown by thin layer chromatography (TLC) and gas chromatography (GLC). The melting points in Figure 1 had been determined on a Kofler Micro Hot Stage using a heating rate of 1-2 C/min. They have also been determined in a Mettler FP2 apparatus with a rate of 0.2 C/min.

[1]Parts I, II and III of this series have appeared in Arkiv Kemi 31:267, 283, 291 (1969).

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The values obtained were generally lower, on the average 0.8 C.

Powder photographs have been recorded with a Guinier type camera and also during continuously varying temperature in a so-called DPT camera (Diffraction Pattern-Temperature) described by Stenhagen (14).

IR spectra have been recorded with a Perkin-Elmer Model 157 spectrophotometer. The substances were mixed with freeze-dried potassium bromide in an agate mortar and pressed to pellets with cold tools. This gentle mixing avoids destruction of the sample crystals, known to occur by prolonged treatment of samples with crystalline potassium bromide in vibration mills. With the present kind of pellet preparation, the crystal form is not altered as shown by a comparison of potassium bromide and Nujol mull spectra of polymorphic long chain fatty acids (15). During the first recording, the temperature in the pellet was kept at about 25 C by air cooling. In many cases spectra have also been recorded at higher temperatures and after melting and cooling. The instrument is linear in wavelength, but in the text wave numbers will be used. According to the specification, the accuracy in wave numbers is ± 2 cm^{-1} and the resolution 5 cm^{-1} at 1000 cm^{-1}.

X-RAY STUDIES

The Guinier photographs showed side spacings characteristic of β'-forms according to the nomenclature of Larsson (16). This was confirmed by the IR spectra and means that the chain packing is of the orthorhombic type with every other chain plane almost perpendicular to the others. Due to strong orientation effects in the specimens, no long-spacing lines were obtained.

The DPT photographs showed no phase transitions during heating. The phase crystallized from melt always showed the same long-spacing as the original solvent-crystallized specimens. The intensities of the lines could differ much for specimens differently solidified, but this difference is probably caused by orientation effects, and it thus seems that all the substances occur in only one form. This was also the case for esters of even alcohols investigated by Lutz et al. (6).

The values of long-spacings obtained with the DPT camera are not so accurate as those of Lutz and coworkers. For the nine substances common to both studies the greatest deviation

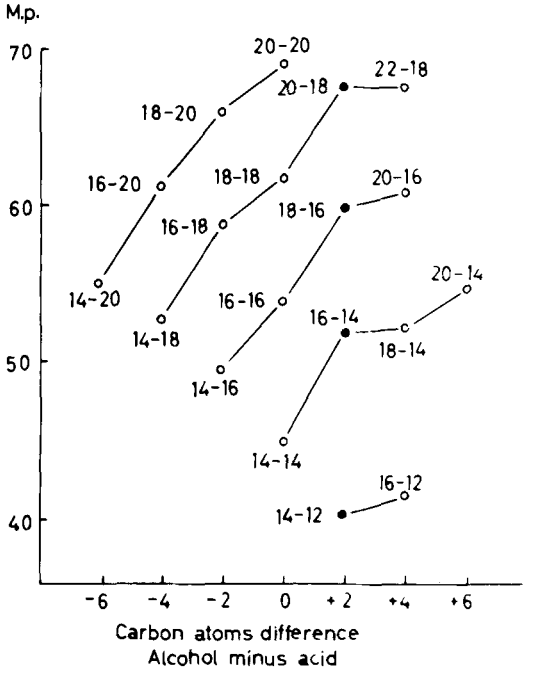

FIG. 1. Melting points of saturated wax esters (13). The figures denote the number of carbon atoms in the alcohol and acid part respectively.

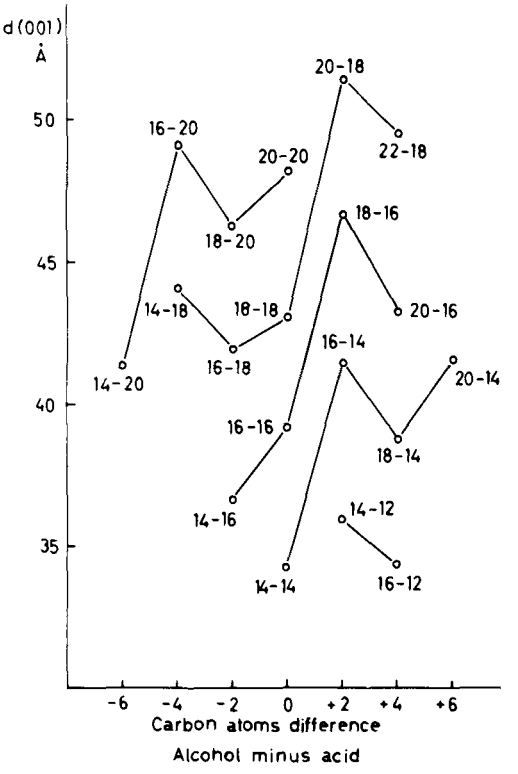

FIG. 2. Long-spacings of saturated wax esters. The figures denote the number of carbon atoms in the alcohol and acid part respectively.

TABLE I

Long-spacings (A) of Wax Esters

Ester	Type[a]	Present study	Reference 6	Reference 5[b]
Tetradecyl dodecanoate	V	36.0		
Hexadecyl dodecanoate	T	34.4		
Tetradecyl tetradecanoate	T	34.3	34.54 ± 0.06	
Hexadecyl tetradecanoate	V	41.5	41.36 ± 0.04	
Octadecyl tetradecanoate	T	38.8	38.98 ± 0.02	
Eicosyl tetradecanoate	T	41.6		
Tetradecyl hexadecanoate	T	36.7	36.79 ± 0.01	37.0
Hexadecyl hexadecanoate	T	39.2	38.90 ± 0.01	41.2 40.7
Octadecyl hexadecanoate	V	46.7	45.98 ± 0.01	46.1
Eicosyl hexadecanoate	T	43.3		
Tetradecyl octadecanoate	V	44.1	43.44 ± 0.01	43.5 (39.3)
Hexadecyl octadecanoate	T	42.0	41.34 ± 0.02	41.4 41.8
Octadecyl octadecanoate	T	43.1	43.47 ± 0.02	43.7
Eicosyl octadecanoate	V	51.4		
Docosyl octadecanoate	T	49.5		50.2 (53.9) 54.3 (50.7)
Tetradecyl eicosanoate	T	41.4		
Hexadecyl eicosanoate	V	49.1		
Octadecyl eicosanoate	T	46.3		
Eicosyl eicosanoate	T	48.2		

[a]V, vertical; T, tilted.

[b]Values in parenthesis denote a second set of (weaker) long-spacing lines. Values in two rows denote different preparations.

is 0.7 A. Our values are given in Table I together with those of Kreger and Schamhart (5) and Lutz et al. (6). When our values are plotted against the carbon atom difference as in Figure 1 the diagram in Figure 2 is obtained. For the difference +2 the same kind of deviation is visible as in the melting point diagram but there is also a longer long-spacing for the difference -4. The latter feature has no counterpart in the melting point diagram in Figure 1.

When comparing the observed long-spacings with values calculated for straight molecules one finds that those with the longer spacings are of the vertical type (marked with V in Table I) while the others are tilted (marked with T in Table I). If the molecules are supposed to be straight also in this phase, the angle of tilt is close to the value 63 ° found for other esters (17-19).

INFRARED SPECTRA

The spectra have been recorded from 4000 to 700 cm^{-1}, and the interval 1500 to 700 cm^{-1} is presented in Figures 3-7, in each of which the acid part is the same. In Figure 8 the spectra of the palmityl esters are shown to illustrate the effect of varying the acid chain length.

The bands in the spectrum of methyl octadecanoate have been assigned to vibrations extensively by Susi and Jahn (20) from measurements with polarized light and the spectrum has also been discussed in connection with the spectra of the homologous methyl alkanoates (21).

The assignment of the bands in methyl octadecanoate is as follows (wave numbers from Reference 20). The antisymmetric stretching of the methylene groups gives rise to a band at 2925/2920 cm^{-1} while the symmetric stretching appears at 2853/2850 cm^{-1}. The average values obtained for the wax esters are 2914 and 2852 cm^{-1}, respectively. The carbonyl stretching frequency (1742 cm^{-1}) is found at 1731 cm^{-1}. The methylene bending mode (1474/1464 cm^{-1}) is obtained at 1466 cm^{-1}, in some cases with an inflection on the high frequency side. The α-CH_2 bending (1413 cm^{-1}) has been found at 1414 cm^{-1}. The symmetric methyl bending frequency (1380 cm^{-1}) absorbs at 1398 or 1375 cm^{-1}. The latter band has possibly the highest contribution of methyl bending as it disturbs the regular progression pattern.

The band progression caused by the methylene wagging modes (23,24) between about 1380 and 1170 cm^{-1} has been observed to be independent of the length of the alcohol chain in accordance with References 2 and 3. The number of strong peaks between 1330 and 1170 cm^{-1} are for the dodecanoates 6, tetradecanoates 7, hexadecanoates 8, octadecanoates 9 and eicosanoates 10. This is clearly demonstrated in Figure 8. Towards higher wavenumbers two groups of weaker bands appear, the first at 1415 and 1398 cm^{-1}, the second at about 1375 and 1362 cm^{-1}. The latter group is surrounded with more inflections in the longer chain compounds than in the shorter ones. The band at 1375 cm^{-1} is the most intensive one, and probably methyl symmetric bending makes a large contribution to it.

In the region 1150 to 950 cm^{-1} where the C-C stretching vibrations absorb, the spectra show differences both within the series shown in Figures 3-7 and between the series. Here, obviously, the O-alkyl chain has an influence on the spectra. One band around 1099 cm^{-1} and two strong bands at 961 cm^{-1} and 922 cm^{-1} are found in all spectra. They are quite strong and thus probably due to C-C stretching near the ester group or $O\text{-}CH_2$ stretching. $O\text{-}CH_2$ stretching was found by normal coordinate analysis to absorb in that region (846 cm^{-1}) (25). These bands are also prominent in the higher members of the series of octadecanoates described in Reference 2.

A band constant for all spectra is found at 891 cm^{-1}, possibly caused by methyl rocking vibrations. A corresponding band was reported for methyl to octyl stearate (2) where also a band at 879 cm^{-1} occurs. Between about 820 and 740 cm^{-1} there are some weak peaks or inflections caused by methylene rocking vibration. Their positions differ between the series and are the same within the series. In some spectra an extra peak or inflection was observed without any evident relation to chain length. At 730 and 720 cm^{-1} are the two strong methylene rocking peaks characteristic of the chain packings with two sets of nearly perpendicular chain planes [β'-forms according to Larsson (16)].

DISCUSSION

Present studies support the conclusion that in series of wax esters the occurrence of abnormally high melting points--when alkyl is 2 carbons longer than acyl (+2 type)--is correlated with the occurrence of vertical crystal forms. However, esters with the alcohol chain 4 carbon atoms shorter than the acid chain (-4 type) also are vertical but they do not show high melting points. The vertical phase here seems to have about the same stability as the tilted, and Kreger and Schamhart (5) found both forms for tetradecyl octadecanoate.

A comparison with dielectric data in References 8-12 shows no correlation between the occurrence of rotation and vertical crystal forms. The latter are obviously not of α-type (in which the molecules have considerable orientational freedom around the long axis). This is also in accordance with the x-ray and IR data which in all cases indicate β'-forms.

It is interesting to note that in some cases the dielectric studies (8,9) show transitions between rotating and nonrotating forms. The rotating form often supercools. On heating, the transitions are sharp. The dielectric behavior also depends on the freezing rate. The x-ray studies showed no change in pattern which could be correlated with reported rotational transitions.

The IR spectra show no obvious differences between vertical and tilted forms or between rotating and nonrotating forms. This may be taken as a confirmation of the view expressed by Crowe and Smyth (8) and Hunter and Eddy (12) that the molecules rotate as a whole so that the molecular geometry is not changed and does not cause marked changes in the IR spectra.

For most substances the spectra have been recorded also during cooling of the melted specimen. As judged from the appearance of the 720 cm^{-1} region a β'-form (with a double peak) is formed directly from the melt.

Tetradecyl octadecanoate, however, showed a spectrum with a single peak at 720 cm^{-1} while the rest of the spectrum (recorded earlier) indicated a solid substance. The DPT photographs showed no changes below the freezing point. The substance was found to be nonrotating by Crowe and Smyth (8) and Crowe et al. (9) and their curves indicate no transition. An explanation might be that solidification is not a simple process in that each individual molecule could be in the same all-*trans* configuration as in the solid, thereby giving mostly the same spectrum as solid, but that the chain packing is not yet ordered in the orthorhombic way necessary for giving a doublet at 720 cm^{-1}. More detailed experiments with accurate temperature control are needed to clarify the situation.

The IR spectra give information as to the chain length of the acid part, in that the number of progression bands between about 1330 and 1170 cm^{-1} is half the number of carbon atoms in the acid. The O-alkyl chain modes are obviously not intensified in the same

way as those of the C-bonded chain, and changes in the length of the alcohol chain make only small differences in the spectra, mainly in the region of skeletal vibrations at 1150 to 950 cm^{-1}. From the appearance of this region and the number of progression bands between 1330 and 1170 cm^{-1} it should be possible to identify these wax esters when reference spectra are available.

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Deposition of Cyclopropenoids in the Tissue Lipids of Rainbow Trout Fed Methyl Sterculate[1]

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ABSTRACT

Groups of young rainbow trout were fed diets containing 0, 100 and 200 ppm methyl sterculate. Their tissue lipids were analyzed for cyclopropene content using the modified Halphen test. Halphen-positive compounds, calculated as sterculic acid, accumulated in all tissues analyzed and reached concentrations approximately equal to those of the dietary lipid. Gas liquid chromatography used in conjuction with mild hydrogenation of the cyclopropene ring revealed that these Halphen-positive compounds were predominantly, if not entirely, sterculic acid which was esterified in the triglycerides. To facilitate analysis of cyclopropenes in liver tissue, a method was developed whereby interfering pigments were removed prior to the Halphen test.

INTRODUCTION

Cyclopropenoid fatty acids (CPFA) occur naturally in the lipids of plants in the order Malvales (1). The most nutritionally and economically important of these lipids is cottonseed oil which contains small quantities of both malvalic acid, ω-(2-*n*-octylcycloprop-1-enyl) heptanoic acid, and the 19 carbon homolog, sterculic acid.

These acids have been studied extensively because of their implications in a variety of physiological disorders (2). In work directly related to this study, Sinnhuber et al. (3) and Lee et al. (4) found that cyclopropenoid fatty acids fed as *Sterculia foetida* oil greatly increased the incidence and growth of aflatoxin-induced hepatomas in rainbow trout. Although these acids were not carcinogenic themselves, Roehm et al. (5) have shown that liver biochemistry and histology is greatly changed with dietary CPFA levels as low as 10 ppm. A possible explanation for the biological activity of CPFA is that sulfhydryl groups add rapidly to the highly strained double bond of the cyclopropene ring (6). A limited number of enzymes known to contain sulfhydryls have, in fact, been inhibited in this manner in vitro (6-8). In spite of the fact that the cyclopropene ring is acid labile, it has been found in the tissue lipid of some laboratory animals (9) fed CPFA. Only the presence of the ring, however, has been shown and the compound itself has not been identified.

These experiments were undertaken to document the level of cyclopropene present in trout tissue during and after the feeding of sterculic acid. The cyclopropene containing compound in the tissue was also tentatively identified.

MATERIALS AND METHODS

Feeding Studies

The fish used in this study were Mt. Shasta strain rainbow trout (*Salmo gairdneri*) which had been artificially spawned and maintained in fiberglass tanks at a temperature of 11.4 C. These trout were fed a semipurified diet described previously (10) which contained methyl sterculate added into the dietary lipid (lipid was 10% salmon oil rather than the 5% corn oil and 5% salmon oil originally described). The methyl sterculate was purified from *Sterculia foetida* beans using the procedure of Kircher (6) which employs urea adduction and low temperature crystallization. An ester sample was obtained which contained 96.48% methyl sterculate, 1.65% methyl malvalate and 1.87% methyl linoleate.

Duplicate lots of 150 fish each were fed diets containing 0, 100 or 200 ppm CPFA for a period of 200 days. After 100 days of this feeding period, 25 fish from each lot fed 200 ppm CPFA were tagged and placed on the control diet. At regular intervals 10 fish were randomly selected from each group and killed by exposure to concentrated tricane methane sulfonate. Samples of liver, muscle and adipose tissue were removed for analysis.

Analysis of CPFA

Lipids were extracted from the tissue using

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[3]This paper represents part of the thesis research conducted at Oregon State University in partial fulfillment for the degree of Doctor of Philosophy.

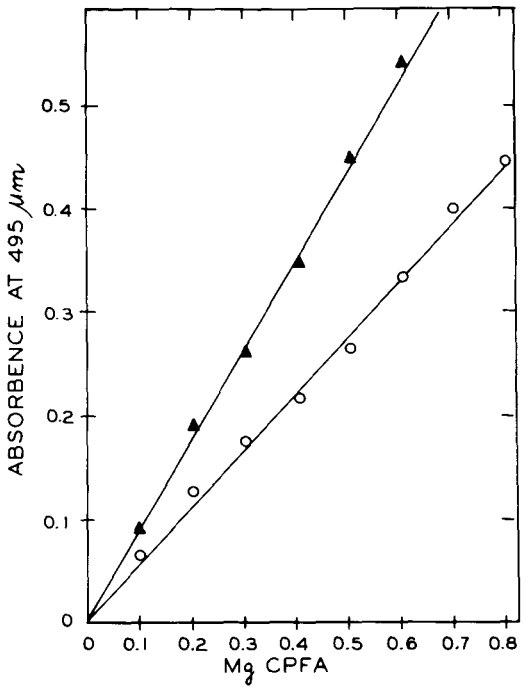

FIG. 1. Halphen-color intensity vs. concentration of methyl sterculate; ○, 25 ml dilution; ▲, 10 ml dilution.

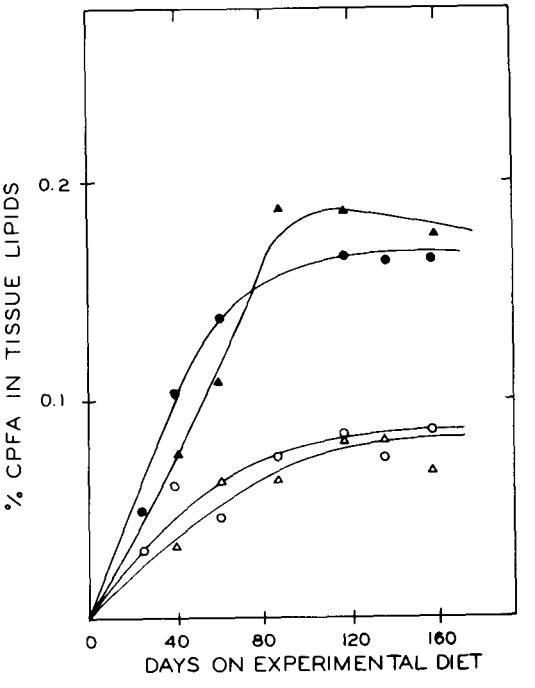

FIG. 2. Accumulation of CPFA in the muscle and depot lipid of trout. Muscle (▲) and depot (●) lipids from fish fed 200 ppm CPFA. Muscle (△) and depot (○) lipids from fish fed 100 ppm CPFA.

the method of Folch (11) and cyclopropenes were analyzed spectrophotometrically using a modification of the Halphen test (12). This method lacks precision and uses a complex and poorly understood reaction, but was selected on the basis of its high sensitivity. The lipid to be analyzed was dissolved in 10 ml butanol and 2.5 ml of 1% sulfur in carbon disulfide, and heated 2 hr at 110 C in 25 ml low actinic volumetric flasks with foil covers. The foil covers were then removed and the heating continued 30 min. The solution was then cooled under tap water, diluted with either 10 or 25 ml butanol, and the absorbance at 495 mμ recorded immediately using a Beckman DU spectrophotometer. Each analysis was done in triplicate.

This determination was quantified using a standard curve obtained from serial dilutions of purified methyl sterculate, and the results were, therefore, calculated as per cent sterculic acid. It should be noted, however, that the Halphen test is specific only for the cyclopropene moiety and other procedures are necessary to identify the actual compound. Two standard curves obtained in this manner are shown in Figure 1. The two curves are identical treatments except for the final dilution with butanol. As can be seen, both curves follow the Beer-Lambert Law, but they cannot be used interchangeably on the basis of concentration of CPFA (i.e., mg CPFA/ml butanol). A new standard curve must be obtained for each dilution of reactants and reagents used.

When this test was used to analyze liver lipids, interfering orange pigments were produced which resulted in extremely high and variable readings. To remove these interfering substances the lipid was first interesterified using a sodium methoxide catalyst to form methyl esters. These esters were then applied in hexane to a small glass column containing 5 g silicic acid which had been activated at 110 C and equilibrated with 4% water as described by Luddy et al. (13). The esters were completely eluted with 150 ml of 1% diethyl ether in hexane and were suitable for analysis using the modified Halphen test.

The liver lipid was also separated into polar and nonpolar lipids using silicic acid column chromatography. The column used was similar to that used for the ester cleanup except that the silicic acid was not equilibrated with water. Nonpolar lipids, primarily triglycerides, were eluted with 150 ml of chloroform, and polar lipids were eluted with the same volume of

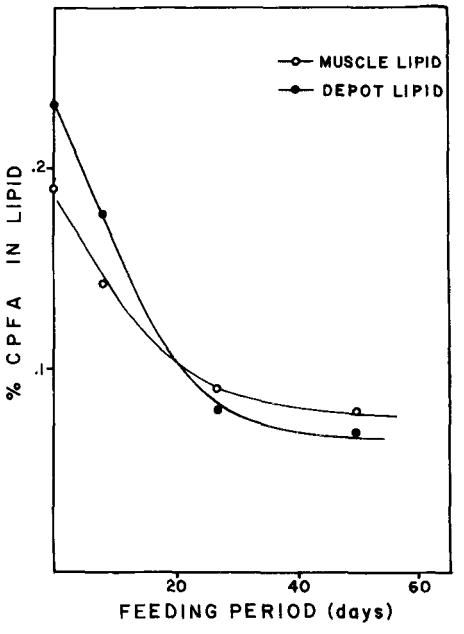

FIG. 3. The CPFA concentration of muscle and depot lipids from trout fed 200 ppm methyl sterculate for 100 days and then fed a CPFA-free diet. The feeding period shown refers to the CPFA-free diet.

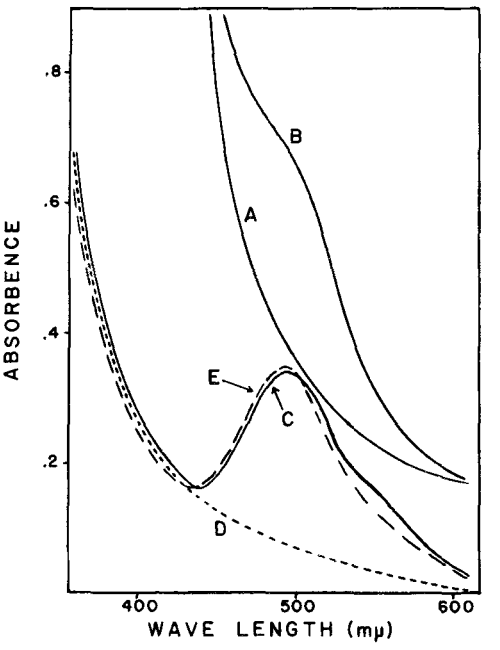

FIG. 4. Absorption spectra of Halphen pigment in trout lipids. (A) control liver lipid; (B) liver lipid + CPFA; (C) liver lipid + CPFA + cleanup; (D) depot lipid; (E) depot lipid + CPFA.

methanol. After chromatography, the interfering pigments were still present in the nonpolar lipids.

In addition to analysis of the cyclopropene ring, methyl sterculate was determined in the purified esters, diet and tissue lipids using a combination of hydrogenation and GLC. The lipid was first partially hydrogenated with 5% palladium on $BaSO_4$ which completely saturated the cyclopropene ring with a minimum of ring cleavage (<4%) to straight chained and branched products. Some polyenoic esters were also partially hydrogenated, but no conversion of monoenes to saturated esters occurred. The saturated esters, including the cyclopropanes, were separated from the unsaturated esters using argentation column chromatography according to the method of DeVries (14). These esters were then analyzed by gas liquid chromatography (GLC) as described previously (5).

Since the ratio of saturated straight chain acids to cyclopropanoid fatty acids remained unchanged during this procedure the concentration of corresponding cyclopropenes in the original lipid could be calculated. The silver ion chromatography also effected a concentration of the saturated esters by removing unsaturates and allowing analysis of smaller quantities of CPFA.

RESULTS AND DISCUSSION

The ingestion of CPFA by trout resulted in an accumulation of compounds containing the intact cyclopropene ring as determined by the Halphen test. These results are shown in Figure 2. With both the intermuscular and depot lipids, this accumulation was gradual and approached a maximum after approximately 100 days. The concentration of CPFA was identical in both the muscle and depot lipids, and appeared to approach the dietary lipid level (i.e., 0.1% and 0.2%, respectively). In both cases, the tissue lipid concentration ofCPFA reached a maximum at approximately 82% of the dietary lipid concentration.

When fish which had been fed the 200 ppm CPFA diet for 100 days were placed on the control diet, the CPFA content of the tissue lipids decreased rapidly (Fig. 3). This rate of decrease was reduced, however, after approximately 30 days and levels of CPFA somewhat less than half the original concentration remained in the tissue after 50 days on a CPFA-free diet.

The determination of liver lipid cyclopropenoids was found to be quite difficult due to the small quantities of lipids available for analysis. For this reason, routine analysis such as was carried out with muscle and depot lipids

was not possible. On several occasions throughout the experiment, however, liver samples were pooled and the lipids were analyzed for CPFA.

The lipid cleanup procedure developed to eliminate interfering pigments was checked for quantitative and qualitative accuracy. Lipids containing internal standards of identical amounts of methyl sterculate were taken through various stages of the procedure and the absorption spectra of the Halphen test product was determined (Fig. 4). Normal trout depot lipid was selected as the blank control, and produced the spectrum shown in curve D. Depot lipid containing 0.274% CPFA as an internal standard showed a clearly defined absorption maxima at 495 mμ (curve E) and with the background subtracted had an absorbance of 0.280 A. Normal liver lipid (curve A) had an abnormally high background of 0.380 A at 495 mμ. This high background was found to vary considerably among liver lipid samples and was independent of the amount of CPFA present. When 0.274% of purified methyl sterculate was added to this oil, its absorption spectrum (curve B) had a large shoulder at 495 mμ and an absorbance of 0.700 A. No clearly defined peak could be seen.

An oil identical to that shown in spectrum B was taken through the cleanup procedure and its spectrum (curve C) was almost identical to that observed with the spiked depot lipid (curve E). A small shoulder appeared at 550 mμ which was not observed in any of the other samples. This shoulder corresponds to that reported by Bailey et al. (12) which occurred in refined cottonseed oil but not in the crude oil. Addition of phosphatides to the refined oil suppressed its formation. The complete removal of phosphatides in our cleanup procedure most likely accounts for this similar observation. The reduction of absorbance after the cleanup procedure (curve C vs. E) was calculated at 5.3% and indicates that no significant destruction of CPFA occurs during base-catalyzed interesterification with 0.1 N sodium methoxide or during short-term silicic acid chromatography.

The liver lipids accumulated CPFA much more rapidly than the depot lipid and muscle lipid and reached a maximum concentration at the first sampling (10 days). The cyclopropenes found in the liver triglycerides were present in concentrations almost identical to that of the respective dietary lipid. The liver triglycerides from trout fed diets having lipid concentrations of 0.10% and 0.20% CPFA contained 0.104% and 0.214% CPFA, respectively. No cyclopropenes were found in the liver phospholipids.

Using the GLC technique described it was ascertained that at least the great majority of cyclopropenoids detected in trout lipid were present as sterculic acid. Table I shows a comparison between total CPFA values found with the Halphen test and sterculic acid which was analyzed by GLC as dihydrosterculic acid after hydrogenation and silver ion chromatography. Due to the inherent quantitative inaccuracy of this analysis when working with very small concentrations of cyclopropenes, it was not possible to determine the extact percentage of Halphen-positive compounds present as sterculic acid. No other unusual compounds were found, however, quantitative analysis did reveal that sterculic acid was present in concentrations near that predicted by the Halphen test.

The data obtained in this study show that sterculic acid, and presumably other cyclopropenoids, fed to rainbow trout, accumulates in body triglycerides as the intact cyclopropenoid. Evidence for a lengthy biological half-life for cyclopropenoids is the fact that after 30 days on a CPFA-free diet slightly less than half the original tissue level of CPFA still remained. During this period the fish gained in weight from approximately 27 g to 42 g. A dilution effect from new tissue lipid formed during this period could, therefore, account for much of this apparent loss. When one considers the reported reactivity of the cyclopropene ring (1,6) the retention of the intact ring in vivo is surprising. The observation that intact CPFA was incorporated into triglycerides and was not found in phospholipids may really be a reflection of increased stability of the cyclopropene ring when incorporated into a triglyceride.

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TABLE I

Comparison of Halphen-Color Response to GLC Analysis of Sterculic Acid in Tissues of Trout Fed a Diet Containing 200 ppm for 117 Days

Tissue	% CPFA (halphen +)	% Sterculic acid (GLC analysis)
Depot lipid	0.167	0.155
Muscle lipid	0.187	0.167
Liver triglycerides	0.214	0.197

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A Possible Block in the Intermediary Metabolism of Glucose Into Proteins and Lipids in the Brains of Undernourished Rats

ABSTRACT

[U-^{14}C] Glucose or [1-^{14}C] L-leucine was injected intraperitoneally into 28-day-old undernourished rats and control sibs who were killed 6 hr later. Brain proteins and lipids were extracted and the lipids fractionated by silicic acid column chromatography into cholesterol, glycolipids and phospholipids. The specific activity of labeled carbon derived from [U-^{14}C] glucose in brain proteins was reduced by 25% in undernourished animals when compared to controls. A similar reduction was seen in the specific activity of brain lipids of undernourished animals: 14% for cholesterol, 21% for phospholipids and 35% for glycolipids. When [1-^{14}C] L-leucine was used as a direct precursor of brain protein synthesis, the specific activity in the undernourished group was only 5% less than that found for the controls. This was not statistically significant. The results suggest that there may be a block in the intermediary metabolism of glucose in the brains of undernourished rats that reduces the availability of glucose carbon to the precursor pool used for protein and lipid synthesis.

The rapid flux of [U-^{14}C] glucose into glutamate and related amino acids as well as into precursors of lipids in the adult nervous system has been well documented (1-3). As a result, [U-^{14}C] glucose is a useful isotope for labeling both proteins and lipids in nervous tissues (4-6). In this communication we present evidence that in undernourished rats there is a block in the incorporation of labeled carbon derived from glucose into brain proteins and lipids.

Sprague-Dawley rats were undernourished from birth to 28 days of age by being placed in a litter of 20 to 22 pups with one mother whereas sibs were raised in litters of 6 to 8 animals. Twenty-eight-day-old undernourished rats and control sibs were injected intraperitoneally (0.25 μc/g body weight) with either [U-^{14}C] glucose (5 mc/mMole) or [1-^{14}C] L-leucine (28.1 mc/mMole). The animals were killed 6 hr later by decapitation and the brains removed and frozen between two pieces of dry ice. The method of Vrba et al. (5) was used for the preparation of total proteins and lipids. Protein was estimated by the method of Lowry et al. (7). The lipid extract obtained by Vrba's method was washed as described by Folch et al. (8). Lipid classes were then separated into three fractions, neutral lipids, glycolipids and phospholipids, by silicic acid column chromatography as described by Rouser et al. (9). Cholesterol was measured by the method of Zlatkis et al. (10), galactose by the method of Hess and Lewin (11), and lipid phosphorus by the method of Rouser et al. (12). The protein samples used for measurement of radioactivity was prepared as described by Agrawal et al. (13) and counted in a Nuclear Chicago spectrometer at an efficiency of 87%. The lipids in each fraction were dissolved in chloroform-methanol (2:1 v/v) and counted in fluid containing 4.0 g Omnifluor in 1000 ml toluene at an efficiency of 90%. The counting efficiency for lipids was determined both by the method of an external standard ratio while an internal standard (^{14}C toluene) was used for counting proteins.

Body and brain weights of the undernourished rats at 28 days of age were reduced by approsimately 45% and 13%, respectively (Table I). The incorporation of ^{14}C metabolites derived from [U-^{14}C] glucose into the brain protein of undernourished animals was inhibited by 25% compared to controls, whereas the incorporation into phospholipids was decreased by 21%, glycolipids by 35%, and cholesterol by 14% (Table I). With the exception of cholesterol, the decrease in incorporation of ^{14}C into protein and lipid fractions in the undernourished rat was statistically significant at a level of $p<0.05$.

When these experiments were repeated with [1-^{14}C] L-leucine, a direct precursor of the proteins, the rate of incorporation of [1-^{14}C] L-leucine into brain proteins of poorly

TABLE I

Incorporation of [^{14}C] Precursors Into Brain Proteins and Lipids of 28-Day-Old Undernourished and Control Rats[a]

	Precursor	Undernourished	Control	Inhibition (%)	p Values
Body weight, g	[U-^{14}C] Glucose	41.14 ± 7.39 (9)[b]	81.40 ± 9.26 (8)	49	<.001
	[1-^{14}C] Leucine	52.87 ± 8.54 (6)	91.88 ± 6.00 (6)	42.4	<.001
Brain weight, g	[U-^{14}C] Glucose	1.373 ± 0.091 (9)	1.569 ± 0.085 (8)	12.4	<.001
	[1-^{14}C] Leucine	1.410 ± 0.082 (6)	1.647 ± 0.068 (6)	14.4	<.001
Cholesterol, specific activity, cpm/mg	[U-^{14}C] Glucose	747 ± 173 (9)	868 ± 138 (9)	14	Not significant
Glycolipids, specific activity, cpm/mg	[U-^{14}C] Glucose	487 ± 143 (7)	755 ± 215 (7)	34.6	<.05
Phospholipids, specific activity, cpm/mg	[U-^{14}C] Glucose	778 ± 200 (9)	987 ± 192 (9)	21	<.05
Proteins, specific activity, cpm/mg	[U-^{14}C] Glucose	796 ± 236 (9)	1069 ± 218 (8)	25	<.044
	[1-^{14}C] Leucine	1699 ± 226 (5)	1783 ± 268 (5)	5	Not significant

[a]Values are given as the mean ± S.D.
[b]Numbers in parentheses indicate number of animals.

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Since [1-^{14}C] L-leucine is not rapidly metabolized in the small glutamate pool in the brain (14) and is directly incorporated into proteins, a normal rate of incorporation of the compound into brain proteins suggests that either protein synthesis per se is not inhibited or that leucine may not be the rate-limiting amino acid controlling the synthesis of protein in the brains of poorly nourished rats. However, a reduced rate of incorporation of ^{14}C metabolites derived from labeled glucose into brain proteins and phospholipids of undernourished rats strongly suggests a block in the intermediary metabolism of glucose.

The reduced incorporation of [U-^{14}C] glucose into glycolipids could be a manifestation of a true inhibition of cerebroside and sulfatide biosynthesis unless one or more of the steps involved in the conversion of glucose into galactose in the brains of poorly nourished rats is impaired (4). A true inhibition of glycolipid biosynthesis is suggested by an earlier observation of Chase et al. (15) who found that the incorporation of ^{35}S sulfate into sulfatide was reduced in the brains of poorly nourished rats.

If such a block in the intermediary metabolism of glucose eventually reduces the availability of the precursors needed for the synthesis of characteristic membrane constituents, it might exert a deleterious effect both on the biochemical development and the functional integration of the central nervous system.

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Renal Cerebroside in Globoid Cell Leukodystrophy (Krabbe's Disease)

ABSTRACT

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The rare, fatal neurological disorder of infants, globoid cell leukodystrophy (GLD), or Krabbe's disease, appears to be caused by a genetic deficiency of (galactocerebroside β-galactosidase galactosylceramide-galacotsyl hydrolase)(1). We demonstrated the enzyme

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TABLE I

Renal Cerebrosides in Globoid Cell Leukodystrophy and Controls

	Age, months	Water content, %	Lipid content, % dry wt	Galactocerebroside, mμmoles/g dry wt	Glucocerebroside, mμmoles/g dry wt
Globoid cell leukodystrophy					
1	20	70.3	24.2	426	538
2	24	73.0	33.3	355	333
3	11	77.4	29.3	672	568
4	10	78.9	24.4	597	556
5	21	76.6	26.9	482	678
Mean ± S.D.		75.2 ± 3.5	27.6 ± 3.8	506 ± 128[a]	535 ± 125[b]
Controls					
1	8	78.9	24.8	426	357
2	21	80.2	25.5	376	485
3	16	79.2	27.6	305	269
4	20	78.5	25.8	350	531
5	18	69.9	23.8	283	302
6	19	75.9	28.1	384	356
7	21	80.5	28.6	632	472
Mean ± S.D.		77.6 ± 3.7	26.3 ± 1.8	394 ± 116[a]	396 ± 100[b]

[a]The difference is statistically significant, $0.05 > p > 0.02$.
[b]The difference is statistically significant, $0.01 > p$.

deficiency in the brain, liver, spleen, kidney, serum, leukocytes and cultured fibroblasts (1-3). Unlike other sphingolipid storage disorders, accumulation of the involved lipid, galactocerebroside, in the brain occurs only focally in the characteristic globoid cells, and the total amount is generally far below that of normal brain. To explain this unusual phenomenon, we postulated an early cessation of galactocerebroside biosynthesis due to the disappearance of oligodendroglial cells, the primary site of the galactocerebroside synthesis in the body (1). The clinical and pathological abnormalities of Krabbe's disease are restricted to the nervous system, in which galactocerebroside is present in large amounts. Galactocerebroside is almost absent in most of the systemic organs. However, the kidney normally contains approximately equal amounts of galacto- and glucocerebrosides, although the total amount is much smaller than in the brain (4). The normal presence of galactocerebroside, unaltered histology in Krabbe's disease, and the presence in kidney of profound deficiency of galactocerebroside β-galactosidase make it the organ of choice to examine for possible abnormal accumulation of galactocerebroside in globoid cell leukodystrophy. The results of the analysis of five GLD kidneys, however, somewhat unexpectedly, did not show a specific increase of galactocerebroside.

The kidney samples from five patients with globoid cell leukodystrophy and from carefully age-matched control individuals were kept frozen until analysis. The tissues were extracted and fractionated into major constituents, essentially according to Folch et al (5). The total lipid fraction was subjected to the mercuric chloride-saponification procedure (6), to remove most of the glycerophospholipids. A clean cerebroside fraction was obtained by silicic acid column chromatography (7). The cerebroside fraction was eluted by a mixture of chloroform-methanol (90:10 v/v), and did not contain lactosylceramide. The amounts of galacto- and glucocerebrosides were determined by measuring the content of galactose and glucose of the cerebroside fraction by gas liquid chromatography as trimethylsilyl-ether derivatives. Methanolysis of the cerebroside fraction, extraction of fatty acid methyl esters, and the derivatization to trimethylsilyl ethers were carried out essentially according to Sweeley and Vance (8). The column was a 6 ft stainless steel, packed with 6% JXR coated on Gas-Chrom Q, 80-100 mesh (Applied Science Laboratories, State College, Pa.). Mannitol was used as the internal standard. The relative detector response of manittol to glucose was 1.27 ± 0.09, and that of galactose to glucose was 0.99 ± 0.03, using an electronic integrator..

There were no significant differences between the GLD and control groups in the water content of the tissue or in the proportion of total lipids in the dry weight. The total amount of cerebroside in the control kidneys were approximately 50% higher than those reported by Martensson for "juvenile kidneys" (4). His "juvenile" samples were in the 10- to 17-year-old range. Our control individuals were all less than two years old. Since the content of cerebroside appeared to decrease in older age groups, it may not be surprising to find higher concentrations of cerebrosides in our control specimens. The relative proportion of glucocerebroside to galactocerebroside (1:0.99) was similar to Martensson's data (1:0.88). There was a slight but statistically significant increase in galactocerebroside in the GLD kidneys. However, glucocerebroside was similarly increased in the pathological samples, and the relative amount of these two compounds remained essentially unchanged (1:0.95). Other hexosylceramides were not determined quantitatively, but visual inspection of a thin layer chromatogram of the saponified samples indicated no gross differences between the GLD and control kidneys.

Contrary to the expectation, the data clearly indicated that there is no specific increase of galactocerebroside in the kidneys of patients with globoid cell leukodystrophy, despite the metabolic block in the degradative pathway. The small increase is accompanied by a similar increase in glucocerebroside. There are a few possibilities to explain this finding. One obvious possibility is related to the differences in the relative amounts of galactocerebroside and galactocerebroside β-galactosidase in normal brain and kidney. The enzyme activity is approximately 30% higher in the kidney than in white matter (2), whereas normal white matter contains galactocerebroside which is more than 200 times that of normal kidney. If a similar rate of turnover is assumed for galactocerebroside in these organs, less than 0.5% of the normal concentration of the hydrolase would still be adequate to handle the requirement of normal kidney. The actual residual levels of galactocerebroside β-galactosidase in GLD kidneys were approximately 5% of normal (2). Another possibility is the existence of a regulatory mechanism which shuts off further biosynthesis of galactocerebroside when there is a block in the degradative pathway. There is no experimental evidence for or against this hypothesis, except that such regulatory mechanism does not appear to operate in other sphingo-

lipid storage diseases, in which continued synthesis results in overt accumulation of the involved lipids. This possibility, however, needs to be explored because it also provides an alternative explanation for the lack of overt accumulation of galactocerebroside in the brain.

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Hydrophobic Enzymes in Hydrocarbon Degradation[1]

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ABSTRACT

Pseudomonas aeruginosa (strain 473) constitutively contains an NADP-linked alcohol dehydrogenase. This enzyme is believed to function only in assimilative processes, because growth on primary alcohols, α,ω-diols or *n*-alkanes induces another alcohol dehydrogenase which is not linked to a pyridine nucleotide. The inducible enzyme reduces bovine cytochrome c and various dyes, but not oxygen. At least two variants of the dissimilative NAD(P)-independent enzyme can be induced by choosing the substrate used for growth. The main difference between the two variants is their different capacity to oxidize ethanol. A noteworthy property of the inducible enzyme is its hydrophobic character. Some of its consequences in paraffin dissimilation are discussed. The paraffin hydroxylase system of the heptane-grown *Pseudomonas* was found to hydroxylate various types of hydrocarbons and thus shows a low substrate specificity. On the other hand, remarkable specificities were also encountered; in some cases only the *trans* configuration of a substrate was hydroxylated. With respect to the site of hydroxylation, the enzyme system was quite specific, even to the extent that, in appropriate cases, the hydroxyl group was introduced in the *trans*-position only. The results obtained with the enzymatic hydroxylations, including some of the specificities encountered, can be explained by assuming that substrate molecules capable of attaining a planar conformation are bound by hydrophobic forces to the enzyme surface.

INTRODUCTION

Fundamental studies of paraffin oxidation in the past dealt mainly with dissimilative pathways and aimed at elucidating the reaction mechanism by which the alkane is oxidized to yield an alcohol. The subsequent oxidative reaction leading to the aldehyde was assumed to involve a conventional alcohol dehydrogenase linked to a pyridine nucleotide, similar to those found in other organisms.

In fact, such a pyridine-nucleotide-linked alcohol dehydrogenase was found by various authors (1-3) in cells of their alkane-oxidizing *Pseudomonas* strains. However, we have been able to demonstrate the presence of an additional alcohol dehydrogenase that is not linked to pyridine nucleotides.

An account of these studies (4) will be given, concentrating on the hydrophobic character of the inducible alcohol dehydrogenase and its consequences with respect to metabolic pathways.

In addition, we will show selected results of another study (J.C. Van Ravenswaay Claasen and A.C. Van der Linden, in preparation) of our group on the specificity of the paraffin hydroxylase.

The results obtained by enzymatic hydroxylation of various compounds, including specificities encountered towards *cis* and *trans* configurations, can also be explained by assuming hydrophobic forces in the formation of the enzyme-substrate complex.

EXPERIMENTAL PROCEDURES

All the experiments were carried out with a crude enzyme extract obtained from *Pseudomonas aeruginosa* (strain 473). The extract had been prepared from a sonic homogenate upon centrifugation for 1 hr at 38,000 g. The supernatant liquid contained 10-15 mg protein/ml.

Details on the assay of the alcohol dehydrogenases, either by phenazine-methosulfate-mediated O_2 uptake or by spectrophotometric assessment of dichoroindophenol reduction, have been given earlier (4).

Enzymatic hydroxylations were carried out by vigorously shaking 2 ml of the extract containing 2 mg added NADH with 3 μl of the hydrocarbon substrate in a closed bottle for 1 hr. The products, alcohols and acids, were extracted with ether and the ether was subjected to gas liquid chromatography analysis using a Porapak Q column. Full details will be published elsewhere (J.C. Van Ravenswaay Claasen and A.C. Van der Linden, in preparation).

The rate of the reactions given in Figures

[1]One of five papers being published from the Symposium "Biochemistry of Hydrocarbon Degradation," presented at the AOCS Meeting, Chicago, September 1970.

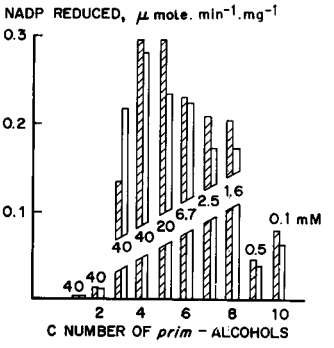

FIG. 1. Specific activity of the NADP-linked constitutive alcohol dehydrogenase with straight primary alcohols. Hatched bars: extract from peptone-glucose-grown cells. Open bars: extract from ethanol-grown cells. Substrate concentration: as indicated in the bars.

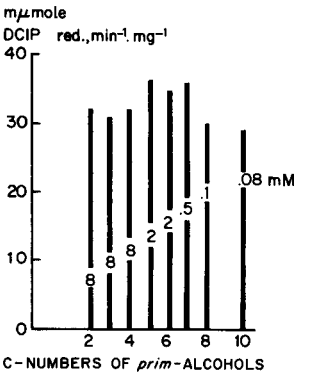

FIG. 2. Rates of 2.6-dichloroindophenol reduction with straight primary alcohols: extract from ethanol-grown cells.

9-12 and 14 was calculated from the total amount of the products recovered. A rate of 100 was arbitrarily set for the highest rate encountered with a *n*-paraffin substrate (*n*-octane). In these figures, the main product is mentioned only. Primary alcohols are often further oxidized by the crude extracts to give the corresponding acids. Cyclic alcohols are not further oxidized.

RESULTS AND DISCUSSION

Alcohol Dehydrogenases

Analysis of extracts of our strain of *Pseudomonas* confirmed the presence of pyridine-nucleotide-linked alcohol dehydrogenases, but unlike the earlier investigators (1-3) we found NADP reduction to be faster than the reduction of NAD. The specificity of our NADP-linked enzyme towards the straight-chain primary alcohols was not unlike that of the NAD-linked enzyme. This specificity is illustrated in Figure 1. With the lower alcohols, in particular with ethanol, the rate of the reaction was low and could not be increased by using substrate concentrations higher than 40 mM. There seems to be no affinity between the lower alcohol and this enzyme. With the higher alcohols the low rate is to be attributed to too low concentrations of the substrate, due to the low solubility of the higher alcohols.

The low rate of ethanol oxidation is surprising, because the cells used in the experiment represented by the open bars were grown on ethanol. Therefore we thought it worthwhile to investigate the crude extracts for the presence of another alcohol dehydrogenase that is more apt to dissimilate the alcohols of various chain lengths. We then found an enzyme which reduces phenazine methosulfate (PMS), dichloroindophenol (DCIP) and cytochrome c, but not NAD(P). PMS is autoxidizable and can be used to mediate between enzyme and oxygen. By use of PMS it can be demonstrated in the Warburg respirometer that the activity of the enzyme depends on the presence of the dye and that it does not react directly with oxygen.

In the next series of experiments we investigated whether this PMS-reducing enzyme was inducible (Table I). Various substrates were used for growing the cells. The activities deter-

TABLE I

Assay of a Constitutive and an Inducible Alcohol Dehydrogenase

Extract, 1 hr, 38,000 g sup. of cells grown on:	Dehydrogenation of 1,6-hexanediol	
	NADP, μmole min^{-1} mg^{-1}	PMS-O_2, μl O_2 h^{-1} mg^{-1}
Peptone-glucose	0.33	2
Hexanedioate	0.23	1
Lactate	0.21	2
1,6-Hexanediol	0.25	73
n-Heptane	0.19	81
Ethanol	0.23	90

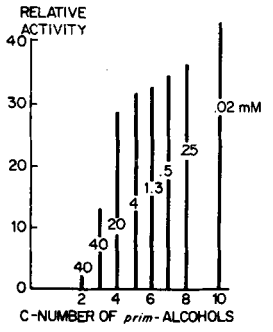

FIG. 3. Rates of 2,6-dichloroindophenol reduction with straight primary alcohols: extract from *n*-octane-grown cells.

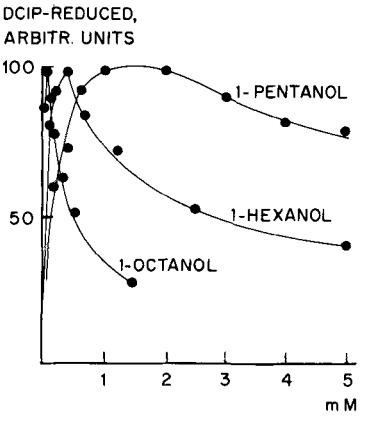

FIG. 4. Inhibition of the inducible alcohol dehydrogenase by excess substrate. Three different extracts of *n*-hexane-grown cells were used. The three curves were corrected to read the same maximum velocity of 100 arbitrary units.

mined by analyzing the extract for the constitutive NADP-linked enzyme merely confirm the constitutive character of this enzyme. All the assays were carried out with 1,6-hexanediol, because this compound had proved to be an excellent substrate for both enzymes. The results of our assay of the PMS-reducing enzyme definitely show that this enzyme is inducible; high values ar found only with the alcoholic growth substrates or with *n*-hexane, which is known to be degraded via an alcohol.

Figure 2 shows the effect of the chain length of the alcohol on its oxidizability by the inducible enzyme as present in extracts of ethanol-grown cells. The assays were carried out by measuring the rate of DCIP reduction. All the oxidation rates from ethanol up to decanol were about equal, but to obtain this result we had, in particular, to adjust the concentrations of the higher alcohols to a value depending on the C-number of the alcohol. An unexpected finding was that results were different when using an extract from *n*-octane-grown cells (Fig. 3). In this case ethanol is not oxidized to any appreciable extent. To obtain a generally consistent result, as in the foregoing experiment, we had to watch the concentrations of the substrate even more carefully.

The higher alcohols were very toxic when overdosed. The concentrations used are written in the bars. They are far below saturation.

When comparing this experiment with the preceding one, we arrive at two conclusions. There are at least two different forms, variants or modifications of the inducible alcohol dehydrogenase. One is capable of oxidizing ethanol, the other is not. The second conclusion is that a regular decrease in optimal substrate concentrations with increasing chain length of the alcohol reflects an increase in affinity of the enzyme for its substrate when the chain length of the alcohol increases.

We continued our studies by looking more closely into the inhibition by excess substrate. Figure 4 shows the enzyme activity as a function of substrate concentration for 1-octanol, 1-hexanol and 1-pentanol. Michaelis' law is obeyed at lower concentrations, but the curves go through a maximum and the rate falls off again at higher concentrations. The decrease in activity at higher concentrations follows Haldane's equation for the inhibition of enzyme action by excess substrate fairly well (Dixon and Webb, 6).

Haldane's equation is based on the assumption that the enzyme has more than one active site and that excess substrate converts an active ES complex into an inactive SES complex. This decreases the amount of the active ES complex, resulting in a decreased rate of enzyme action.

Substrate	Rate[a)]
$HOH_2C \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2OH$	110
$HOOC \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2OH$	4
$H_3CO-C(=O) \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2OH$	110
$HOH_2C \cdot CH_2 \cdot O \cdot CH_2 \cdot CH_2OH$	4
$CH_3 \cdot CH(CH_3) \cdot CH_2OH$	4
$H_3C \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH(CH_3) \cdot CH_2OH$	100

FIG. 5. Oxidation of the primary hydroxyl group of various compounds by an extract of hexane-grown cells. The rate of dichloroindophenol reduction obtained with 1-hexanol is arbitrarily set at 100.

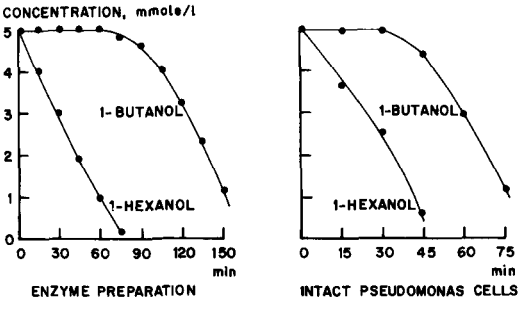

FIG. 6. Competition between 1-hexanol and 1-butanol for the inducible NAD(P)-independent alcohol dehydrogenase. Cells and extract obtained after growth on ethanol. Residual concentrations were followed by GLC analysis of samples taken from the incubation mixture. Phenazine methosulfate added to enzyme preparation.

At higher substrate concentrations Haldane's equation in its reciprocal form, is represented by a straight line.

When we tested Haldane's equation graphically we actually found a straight line, which means that the enzyme has indeed more than one active site, and that the substrate molecule occupies two or more active sites of the enzyme. One of these sites will no doubt be occupied by the hydroxyl group. The other will therefore bind the aliphatic chain. The only way to do so is by formation of hydrophobic bonds between the aliphatic chain and regions of the enzyme surface with a hydrophobic character.

With respect to the hydrophobic bonds, it can be said that they owe their stability to the polarity of the solvent, which is water. Water molecules easily form hydrogen bonds between one another and by doing so they will expel the aliphatic chains, which then tend to stick to hydrophobic parts of the enzyme surface. In

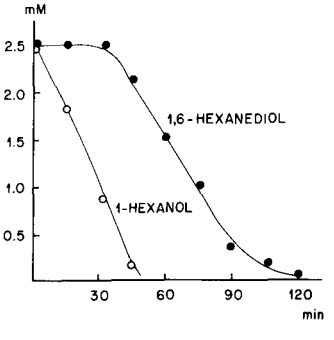

FIG. 7. Oxidation of a mixture of 1-hexanol and 1,6-hexanediol by intact hexane-grown cells. Residual alcohol concentrations were followed by GLC analysis of withdrawn samples.

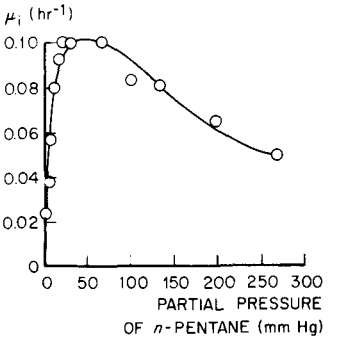

FIG. 8. Effect of partial pressure of *n*-pentane on specific growth rate. Reproduced from Reference 15.

this way it is easy to understand that there will be a negative correlation between the affinity of the enzyme for the alcohol and the solubility of the latter in water.

This negative correlation can be illustrated with the compounds listed in Figure 5.

1,6-Hexanediol is one of the best substrates, because it is not easily overdosed. The corresponding hydroxy acid, which is much more soluble in water, is not a substrate. A high oxidation rate is, however, found for the methyl ester, which has a lower solubility in water. Diethylene glycol is highly soluble in water and is, accordingly, not oxidized, while the same seems to be true for isobutanol when we compare this α-methyl-substituted alcohol with 2-methylhexanol-1.

Another consequence of the hydrophobic character of the enzyme is that it prefers the alcohol with the lower solubility in water, when incubated with a mixture of two alcohols. Figure 6 shows that an extract of ethanol-grown cells, when incubated with equimolar concentrations of 1-hexanol and 1-butanol, first oxidizes 1-hexanol. Interestingly, the results with intact resting cells are quite similar. In both cases 1-butanol is oxidized only upon exhaustion of 1-hexanol.

Figure 7 shows that the extract from hexane-grown cells in a similar experiment selects 1-hexanol from its mixture with 1,6-hexanediol, as can be expected from the lower solubility in water of 1-hexanol.

Our earlier work (6) furnishes another example of a competition between two substrates which, in fact, is a special case of competitive inhibition. During the oxidation of 1,6-hexanediol by resting cells grown on this diol, there is an accumulation of 6-hydroxyhexanoate. Upon exhaustion of the 1,6-diol the enzyme turns to the less hydrophobic 6-hydroxyhexanoic acid, converting it into adipate,

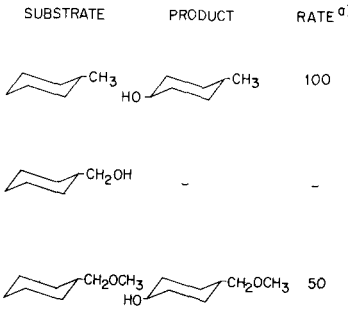

FIG. 9. Enzymatic hydroxylation of monosubstituted cyclohexanes. All rates in this and following figures are expressed as a percentage of the rate of *n*-octane hydroxylation.

which is highly oxidizable and does not accumulate to any appreciable extent. (Free 6-hydroxyhexanoic acid, as already shown in Figure 5, is not a substrate for the inducible alcohol dehydrogenase. In the experiment with intact cells the 6-hydroxyhexanoic acid is converted into its CoA derivative, which has a lower solubility than the free acid.)

From this competitive inhibition it can be concluded that one enzyme is responsible for the oxidation of both the 1,6-diol and the 6-hydroxyhexanoic acid. This experiment and the one of Figure 7 further show that the primary alcohol is oxidized in preference to the α,ω-diol and that in turn the α,ω-diol is oxidized in preference to the ω-hydroxy acid. Again we find that the higher affinity of the enzyme for its substrate correlates with the lower solubility of the substrate in water.

Paraffin Dissimilation. At this point we recall the discussion in earlier years on the question of monoterminal versus diterminal oxidation in paraffin dissimilation. By 1963 a monoterminal attack on the molecule and a pathway through a number of fatty acids linked by 3-oxidation seemed clearly established. However, *in that year* Kester and Foster (7) detected ω-hydroxydecanoic acid in a culture of Corynebacterium on decane and they started a discussion on the question of whether a second pathway, initiated by diterminal attack on the paraffin, could contribute significantly to paraffin dissimilation. This, we learned later, does not seem to be the case.

When looking at that problem now we understand why the ω-hydroxy acid could be detected with relative ease. Our experiments suggest that the hydroxy acid accumulated because of its lower affinity for the alcohol dehydrogenase, which prefers oxidation of the primary alcohol, present during *n*-paraffin dissimilation; the primary alcohol is more hydrophobic than the ω-hydroxy acid. In other words, the presence of a hydrophobic alcohol dehydrogenase will result in a major degradation pathway through the more hydrophobic mono-functional intermediate. Thus α,ω-oxidation products are by-products which will often be easily degradable upon exhaustion of the primary al-

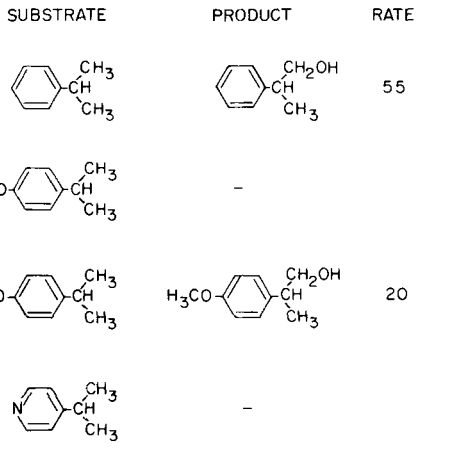

FIG. 10. Enzymatic hydroxylation of substituted benzenes.

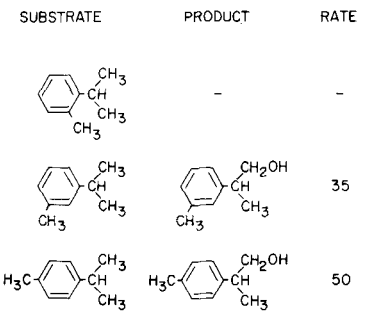

FIG. 11. Enzymatic hydroxylation of cymenes.

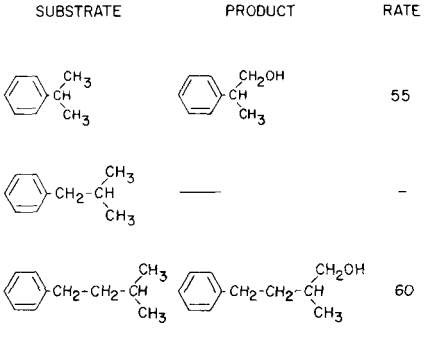

FIG. 12. Enzymatic hydroxylation of alkyl-substituted benzenes.

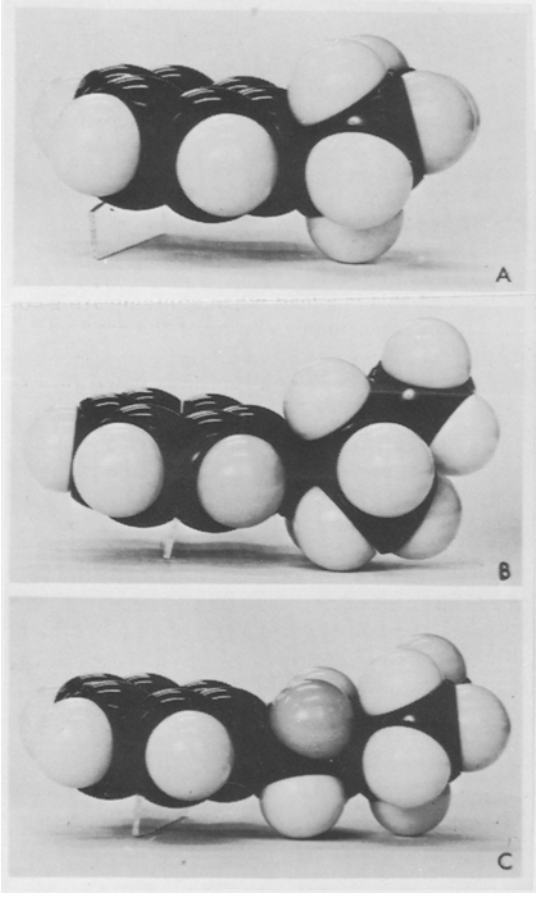

FIG. 13. Scale molecular models of isopropylbenzene (a), isobutylbenzene (b) and isopentylbenzene (c) (see text).

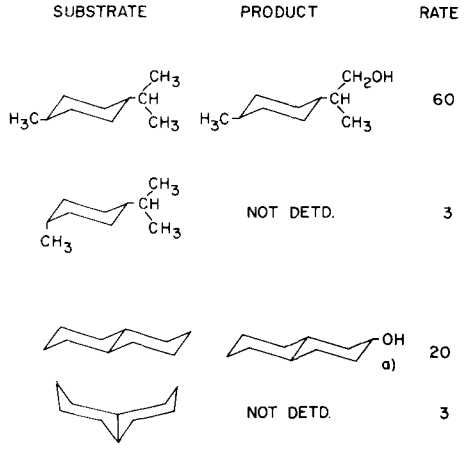

FIG. 14. Enzymatic hydroxylation of *cis*- and *trans*-cyclohexane derivatives. The site of the hydroxyl group is not established.

cohol.

α-Olefin Dissimilation. From investigations on the degradation of α-olefins by bacteria it was concluded that bacteria prefer attacking the olefin at the saturated end of the molecule (8,9), though the double bond was found to be involved in some minor side reactions (10). One of these side reactions was the formation of a 1,2-diol.

A *Candida* yeast, unlike the bacteria, seemed to degrade the α-olefin from the unsaturated end of the molecule, as was concluded by various authors (8,11,12) mainly from the accumulation of the 1,2-diol in the fermentation broth.

Markovetz and his group (13) reinvestigated this yeast and demonstrated that, apart from attack on the double bond, the yeast also hydroxylates the α-olefin at its methyl group, like bacteria.

As far as we know, the main pathway of α-olefin dissimilation in the yeast has not yet been definitely established but we are inclined to assume that the accumulation of the 1,2-diol is again caused by the presence of a more hydrophobic primary alcohol formed by attack at the saturated end of the molecule. In our opinion further experiments might well go to show that α-olefin degradation in yeast and in bacteria is essentially similar and that 1,2-diol formation, as well as other reactions involving the double bond, are more or less important side reactions in bacteria as well as in yeasts.

Paraffin Hydroxylase System

A hydrophobic character of the hydrocarbon oxidizing system is to be expected. Its demonstration as for the alcohol dehydrogenase is far from easy; paraffin concentrations in water cannot easily be adjusted as required. Below we want to review some phenomena which seem to confirm our expectations.

Uemura et al. (14) measured the relation between specific growth rate of a bacterium as a function of *n*-pentane vapour pressure. This relation (Fig. 8) reminds one of the relation found between the activity of the alcohol dehydrogenase and the concentration of *n*-pentanol (Fig. 4). Admittedly, the similarity between the two curves might well be coincidental, but it might also reflect the inhibition of the paraffin hydroxylase by excess substrate.

More convincing arguments can be found when we examine the paraffin hydroxylase for its specificity to hydroxylate various compounds. The examples which follow are a selection from our studies which will be published elsewhere (J.C. Van Ravenswaay Claasen and A.C. Van der Linden, in preparation).

All the hydroxylations were carried out with a crude enzyme extract obtained from *n*-heptane-grown *Pseudomonas* cells. The rate of the reaction was arbitrarily set at 100 for the hydroxylation of *n*-octane. Figure 9 then shows that methylcyclohexane is hydroxylated in the ring at the 4-*trans* position. Cyclohexyl methanol, more soluble in water, is not hydroxylated. Decreased solubility of the methyl ether enables the hydroxylating enzyme system to react again.

A selection of substituted benzene substrates is given in Figure 10. Hydroxylation of the benzene ring was never observed. The isopropyl group, however, easily undergoes hydroxylation at, what is remarkable, one of its methyl groups.

Increasing the solubility by introduction of a phenolic hydroxy group suppresses the hydroxylation completely. If the phenolic compound is methylated, the solubility in water is decreased again. Accordingly, some hydroxylation of *p*-methoxyisopropylbenzene was found. Pyridine compounds are more soluble in water than the corresponding benzenes and the finding that 4-isopropylpyridine is not hydroxylated is not surprising.

We do not want to suggest that the solubility in water is the only decisive factor for the possibility of hydroxylation. Substrate configuration certainly is also important, as follows, e.g., from the hydroxylation of the cymene isomers (Fig. 11). The *o*-methyl group prevents the isopropyl group from becoming coplanar with the benzene ring. It seems possible indeed that the substrate molecule, in order to be hydroxylated, must be able to attain a planar conformation. This is further illustrated with the alkylbenzenes of Figure 12. Molecular models show clearly that isopropyl and isopentylbenzenes, unlike isobutylbenzene, can be forced into relatively planar conformations (Fig. 13).

Two *cis-trans* specificities which can also be explained by the requirement of a flat substrate molecule are given in Figure 14. In both cases the isomers that are capable of attaining a planar conformation are the better substrates for hydroxylation.

In all cases of *cis-trans* specificity encountered in our study, either with respect to *cis-trans* isomers of the substrates, or with respect to those substrates where the hydroxyl group can be introduced into a *cis* or a *trans* position, we found that the isomer favoured is the planar one.

No specificity was noted towards *cis* or *trans*-olefins, which corresponds with the fact that both isomers can attain a flat conformation. Also the absence of specificity noted towards D- and L-methyl-substituted alkanes (15) is in accordance with the view that the substrate is bound to a hydrophobic surface.

Consequently, hydrophobic attachment of the (planar) hydrocarbon to a hydrophobic enzyme surface seems to govern the hydroxylation of growth and of non-growth hydrocarbons, and this is a lead when contemplating co-oxidation experiments aiming at the production of typical compounds.

We realize that our hypothesis based on experiments with heptane-grown cells of *Pseudomonas* 473 and its crude extracts applies to this strain and to this growth-substrate only, but we hope that we have succeeded in demonstrating the importance of hydrophobic interactions for our understanding of dissimilative pathways of aliphatic hydrocarbons as well as for predicting the possibility of hydroxylation of various hydrocarbons and their derivatives by extracts from paraffin-grown cells.

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Metabolism of Alkane by Yeast[1]

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ABSTRACT

We demonstrated two NAD^+-linked alcohol dehydrogenases in cell free extracts of *Candida tropicalis* grown on *n*-tetradecane. Comparative studies of localization, properties and regulation indicate that these enzymes are involved in two different pathways of *n*-alkane metabolism, one cytoplasmic and the other mitochondrial. Kinetic properties, such as the variation of the K_m and V_{max} as a function of substrate chain length of the soluble NAD^+-linked alcohol dehydrogenase, might involve hydrophobic interactions between the substrate and the enzyme.

INTRODUCTION

Aliphatic hydrocarbons are oxidized by way of a primary alcohol and aldehyde to yield the corresponding fatty acid; the exact intermediates in the formation of the primary alcohol have not yet been clearly established. Pathways involving hydroperoxide, alkene and epoxide derivatives have been proposed (1,2). In bacteria a cell free system which hydroxylates hydrocarbons has been shown to involve rubredoxin (3) and two other protein components (4). A bacterial system involving hemoprotein P-450 has also been reported (5). Early studies with yeast have primarily involved a characterization of the oxidation products obtained from cultures of *Candida lypolitica* growing at the expense of 1-alkene (6). Klug and Markovetz (7,8) proposed two pathways for the degradation of 1-alkene involving oxidation at both ends of the molecule. The attack at the double bond was not of minor importance as described by Huybregste and Van der Linden (9) with Pseudomonas species. Recently, Iizuka et al. (10) and Wagner et al. (11) have been able to isolate 1-alkene as a product of enzymatic oxication of alkane by a cell free extract of yeast grown on hydrocarbons. These studies are in accord with the mechanism of hydrocarbon dehydrogenation as reported by Senez and Azoulay (12). Although we have failed (13) to show directly the formation of 1-alkene by cell free extracts of *Candida tropicalis* grown on *n*-alkanes, the formation of 1-alkene as an intermediate in alkane oxidation in these extracts is supported by three facts: (a) There is a decane dependent reduction of NAD^+ in the presence of ATP by cell free extract of tetradecane-grown cells. (b) Induction studies have shown that this NAD^+ reduction is catalyzed by extracts of cells grown on *n*-tetradecane; lower activities are observed with extracts from cells grown on 1-tetradecene. No activity is observed with cells grown on glucose. However, we have observed very low activity with a different strain of yeast grown on glucose. Apparently this organism oxidizes alkane constitutively and has a low level of a constitutive dehydrogenation system (13). (c) Crude extracts of tetradecane-grown cells are able to convert 1-decene to 1-decanol.

This system which catalyzes the decane de-

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TABLE I

Purification of the Soluble NAD^+ Linked Alcohol Dehydrogenase

Fractions	Specific activity[a]	Total activity	Yield, %	$\frac{A_{s_1}}{A_{s_2}}$[b,c]
Sephadex G_{10}	82	108,000	100	1.69
DEAE-cellulose (after precipitation with $(NH_4)_2SO_4$ 80%)	1530	38,000	35	1.58
Sephadex G_{200}	2340	22,000	20	1.64

[a]Specific activity is expressed as nanomoles of NAD^+ reduced per minute per milligram of protein.

[b]A_{s_1} specific activity with 1-decanol as substrate.

[c]A_{s_2} specific activity with 1-10-decanediol as substrate.

TABLE II

Specificity of Substrate of the Soluble Alcohol Dehydrogenase

Substrate	Activity, %
1-Decanol	100
2-Decanol	80
5-Decanol	0
9-Decene-1-ol	42
1-10-Decanediol	50
Ethanol	0
Propanol	0
Butanol	3
Hexanol	25
Cyclohexanol	0
Heptanol	40
Octanol	70
Nonanol	75
Undecanol	85
Dodecanol	120
Tridecanol	90
Tetradecanol	90

pendent NAD^+ reduction in the presence of ATP as well as alcohol and aldehyde dehydrogenases and acyl-CoA synthetase is located in mitochondria (13,14). The mitochondria were prepared from protoplasts obtained from tetradecane-grown cells of *C. tropicalis* (15). We also found a soluble enzyme which was able to carry out the dehydrogenation of primary alcohols. In order to determine whether or not these enzymes are involved in different pathways of degradation of hydrocarbons, we have compared the properties of the mitochondrial and soluble alcohol dehydrogenases.

RESULTS

Study of the Soluble NAD^+-Linked Alcohol Dehydrogenase

The preparation of cell free extracts and the measurement of the enzymatic activity of the mitochondrial alcohol dehydrogenase have been reported elsewhere (13,16). The alcohol dehydrogenase activity is expressed in nanomoles of NAD^+ reduced per minute per milligram protein.

A soluble NAD^+-linked alcohol dehydrogenase (alcohol NAD^+ oxidoreductase EC 1111) has been found in cell free extracts of *C. tropicalis* cultivated on *n*-tetradecane. This enzyme catalyzes the oxidation of 1-decanol and 1-10 decanidiol in the presence of NAD^+. The analysis of the products of the reaction shows that 1-decanol is transformed to decanal and decanoic acid; however, 1-10 decanediol is oxidized only to the corresponding ω-hydroxy-acid.

Purification of the soluble alcohol dehydrogenase has been carried out only in order to study the ratio of the activity measured with 1-decanol and 1-10 decanediol as substrates. Table I shows that this ratio remains constant during the purification.

TABLE III

Determination of K_m and V_{max} of the Soluble Alcohol Dehydrogenase for Primary Alcohols and Diols

Substrate	K_m (m*M*)	V_{max}
1-Decanol	0.24	1750
1-Dodecanol	0.15	1850
1-Tetradecanol	0.12	2125
1-10 Decanediol	0.86	860
1-11 Undecanediol	0.72	940
1-12 Dodecanediol	0.60	1120

With purified extracts the enzymatic activity is proportional to protein concentration. It is specific for NAD^+ (K_m = 0.4 mM) which cannot be replaced by $NADP^+$. The maximal rate of activity is observed at pH 9 in 0.05 M Tris-HCl buffer. The substrate specificity is shown in Table II; 2-decanol is a substrate but other secondary alcohols are not. 1-10 Decanediol as well as 9-decene-1 ol are substrates. It is worth noting that alcohols with a chain length below five atoms of carbon are not substrates of the soluble enzyme.

The Michaelis constants determined with different alcohols are shown in Table III. As a function of the chain length, the affinity of the substrate for the soluble enzyme increases with longer chain lengths. For various alcohols of the same chain length the affinity is higher for the primary alcohol than for the corresponding diol. Maximal velocities increase also as a function of chain length. For the mitochondrial al-

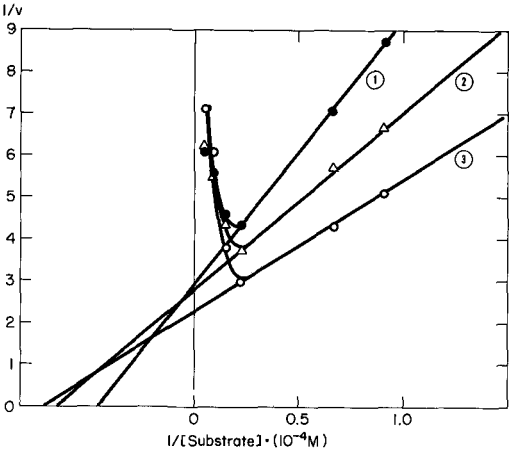

FIG. 1. Variation of K_m (curve 1) and V_{max} (curve 2) as a function of chain length (semilogarithmic graph).

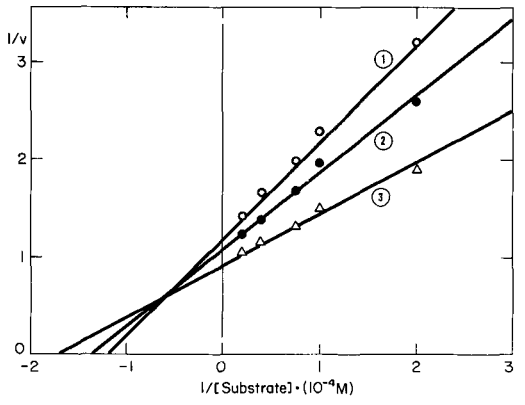

FIG. 2. Reciprocal plot of velocity vs. substrate concentration: 1) 1-decanol, 2) 1-dodecanol, 3) 1-tetradecanol; NAD^+, $7 \cdot 10^{-3}$ M. The system is incubated in Tris-HCl buffer 0.05 M (pH 9) with 1 mg of protein.

cohol-dehydrogenase it has been demonstrated that the affinity of the substrate for the enzyme decreases with increasing chain length. The maximal velocity also increases with chain length (Fig. 1).

The soluble enzyme activity as a function of substrate concentration is shown in Figure 2. Inhibition by substrate is observed at high substrate levels. Inhibition is not observed with diol as substrate (Fig. 3). The kinetics of thermal inactivation, as well as the effects of various inhibitors on the activity, show that decanol and 1-10 decanediol are substrates of the same soluble enzyme. Table IV shows that the activity is not sensitive to KCN or NaN_3. This observation is similar to that previously described by Roche and Azoulay (17) with the alcohol dehydrogenase from *S. cerevisiae* grown on *n*-tetradecane.

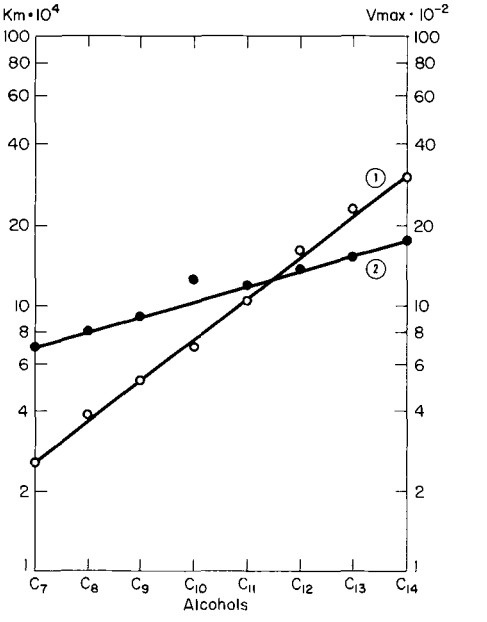

FIG. 3. Reciprocal plot of velocity vs. substrate concentration: 1) 1-10 decandiol; 2) 1-11 undecanediol; 3) 1-12 dodecanediol; NAD^+, $7 \cdot 10^{-3}$ M. The system is incubated in Tris-HCl buffer 0.05 M (pH 9) with 1 mg of protein.

TABLE IV

Effect of Inhibitors on the Alcohol Dehydrogenase Activity Measured With 1-Decanol and 1-10 Decanediol as Substrate

	Inhibition, %	
Inhibitors	1-Decanol	1-10 Decanediol
10^{-3} M		
Iodoacetate	21	20
Hydroxylamine	53	55
KCN	15	13
NaN_3	18	19
$HgCl_2$	100	100
PCMB	100	100
6.10^{-3} M		
n-Decane	46	50
Fluorodecane	59	59
Chlorodecane	39	39
Bromodecane	31	31
Iododecane	23	23

Regulation studies show that the soluble alcohol dehydrogenase is specifically induced by alkane and its metabolic intermediate. No enzyme is detected with glucose-grown cells. In contrast, a low but significant constitutive level of the mitochondrial alcohol dehydrogenase is obtained with glucose-grown cells as shown in Table V.

DISCUSSION

The results obtained demonstrate that both

TABLE V

Influence of the Growth Substrate on the Specific Activities of the Two Alcohol Dehydrogenases

	Specific activity[a]	
Growth substrate	Mitochondrial alcohol dehydrogenase	Soluble alcohol dehydrogenase
Glucose	39	0
n-Tetradecane	600	450
1-Tetradecanol	249	300
Tetradecanal	130	200

[a]Specific activity is expressed in nanomoles of NAD^+ reduced per minute per milligram of protein.

decanol and 1-10 decandiol are dehydrogenated by the same inducible enzyme. However, it is not clear why the decandiol is converted only to the ω-hydroxyacid and not the corresponding dicarboxylic acid. Kinetic studies have shown that the oxidation of the primary alcohols is inhibited by an excess of substrate and that the affinity of the substrate for the enzyme increases with chain length. Van der Linden and Huybregste (18) have previously reported the same properties for an inducible alcohol dehydrogenase which is not dependent on NAD^+ or $NADP^+$. They explained their results by assuming that the enzyme has at least two essential binding sites for the alcohol substrate: one site will bind the hydroxyl group while the other binds the aliphatic chain by hydrophobic bonds. Using such a model and assuming that the minimal distance between the two essential binding sites is at least five carbon atoms, we can easily explain the variation of affinity with different alcohols, the inhibition by excess of substrate, and also the substrate specificity. This model also explains why the diols are oxidized only to the corresponding ω-hydroxyacid, since the polarity of the ω-hydroxyacid does not allow it to fit into the hydrophobic site of the dehydrogenase. A similar model has been proposed by Bardley et al. (19) to explain the kinetic properties and the substrate specificity of the diamine oxidase.

That the soluble alcohol dehydrogenase may result from solubilization of the mitochondrial enzyme is apparently ruled out by the differences in kinetic properties between the two dehydrogenases, although these differences may be due to an altering of the enzyme by solubilization. Induction studies clearly indicate that this soluble alcohol dehydrogenase, different from the mitochondrial enzyme, is involved in another pathway of degradation of hydrocarbons. A second pathway is also shown by the presence in the cytoplasmic fraction of an aldehyde dehydrogenase and a dicarboxylic acyl CoA synthetase (20).

ACKNOWLEDGMENTS

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Microbial Subterminal Oxidation of Alkanes and Alk-1-enes[1]

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ABSTRACT

Oxidation of *n*-alkanes and alk-1-enes by a *Penicillium* species and *Pseudomonas aeruginosa* resulted in the formation of intermediates arising from the oxidation of methylene groups. A catabolic pathway involving oxidation of the hydrocarbon to a secondary alcohol and the corresponding ketone, followed by the formation and subsequent cleavage of an ester intermediate is presented.

Several reports, beginning as early as 1895 (1-4), adequately testify to the fact that various aliphatic hydrocarbons can be assimilated by diverse filamentous fungi. However, the attendant reactions concerned with catabolism of these compounds by fungi have received scant attention. Yamada and Torigoe (5) provided some evidence on the mode of oxidation of aliphatic hydrocarbons. Employing a mixture of *n*-alkanes (C_9-C_{10}) as growth substrate these workers determined that the main acids produced by a *Botrytis* species were the C_9 and C_{10} mono- and dicarboxylic acids derived from the oxidation of nonane and decane in the substrate mixture.

In our laboratory some 53 strains of filamentous fungi representing 32 species were checked for their ability to utilize five even-numbered alkanes and alk-1-enes of 10-18 carbon atoms as sole carbon sources for growth (6). *Cunninghamella blakesleeana*, which exhibited profuse growth on all substrates tested, and a *Penicillium* species were selected for further study using tetradecane and tetradec-1-ene as substrates. The identification of tetradecanoic acid and tetradec-13-enoic acid as predominant acids arising from the oxidation of tetradecane and tetradec-1-ene, respectively, by *C. blakesleeana*, provided evidence for methyl group oxidation (7). However, these results were not seen when the *Penicillium* species oxidized the two C_{14} substrates. Thin layer chromatographic (TLC) analyses of hydrolyzed mycelial mat extracts from cells grown on tetradecane revealed classes of compounds corresponding to secondary alcohols and ketones. Gas liquid chromatographic (GLC) analysis of the alcohol class on a polar column is shown in Table I. The three experimental secondary alcohols corresponded to the retention times of the C_{14} alcohol standards, tetradecan-2-, -3- and -4-ol. Trimethylsilyl ether derivatives of the known and experimental alcohols also compared favorable.

Additional analytical data on the alcohols were furnished by carbon skeleton-hydrogenolytic cleavage procedures (8,9). Analyses of the reduced hydrocarbon fragments obtained after this treatment showed that each alcohol yielded a major carbon fragment of 14, thus indicating chain length, and in addition, minor fragments which arose from reductive cleavage at the functional group were detected, i.e., 10, 11 and 12 fragments (Table II). This was indicative of hydroxyl functions in the 4, 3, and 2 positions,

[1]One of five papers being published from the Symposium "Biochemistry of Hydrocarbon Degradation," presented at the AOCS Meeting, Chicago, September 1970.

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TABLE I

GLC Analyses of Alcohols From Tetradecane[a]

Alcohols	Retention time, min	Trimethylsilyl derivative
Standard		
Tetradecan-4-ol	20.1	5.5
Tetradecan-3-ol	21.2	6.2
Tetradecan-2-ol	23.5	6.7
Experimental		
Tetradecan-4-ol	20.5	5.6
Tetradecan-3-ol	21.6	6.5
Tetradecan-2-ol	24.1	7.1

[a]Analyses were performed at 190 C using a 10 ft column with 5% free fatty acid phase (Varian-Aerograph, Walnut Creek, Calif.) as the liquid phase.

TABLE II

Carbon Skeleton Analyses of Alcohols From Tetradecane

Alcohols	Carbon fragments[a]
Standard	
Tetradecan-4-ol	14 and 10
Tetradecan-3-ol	14 and 11
Tetradecan-2-ol	14 and 12
Experimental	
Tetradecan-4-ol	14 and 10
Tetradecan-3-ol	14 and 11
Tetradecan-2-ol	14 and 12

[a]Hydrocarbon fragments resulting from hydrogenolytic cleavage were analyzed by GLC on an 8 ft, 6% Apiezon L column at 145 C.

TABLE III

GLC Analyses of Ketones from Tetradecane[a]

Compounds	Retention time, min	After reduction
Standard ketones		
Tetradecan-4-one	13.9	
Tetradecan-3-one	15.7	
Tetradecan-2-one	17.9	
Standard alcohols		
Tetradecan-4-ol	21.2	
Tetradecan-3-ol	22.2	
Tetradecan-2-ol	24.2	
Experimental ketones		
Tetradecan-4-one	13.6	21.1
Tetradecan-3-one	16.1	22.5
Tetradecan-2-one	17.7	24.1

[a]Analyses were performed at 185 C, see Table I.

respectively, in the three experimental alcohols.

GLC analysis of the TLC fraction corresponding to ketones is presented in Table III. Retention times of authentic tetradecan-4-, -3-, and -2-ones and the three experimental ketones are given. Reduction of the experimental ketones with sodium borohydride lengthened retention times to correspond to those of known C_{14} secondary alcohols, i.e., tetradecan-4-, -3- and -2-ols. Carbon skeleton-hydrogenolytic cleavage verified that each ketone contained 14 carbons and that the carbonyl function was in the 4, 3 and 2 positions, respectively (Table IV).

The same type of analyses were applied to identification of intermediates from the oxidation of tetradec-1-ene (7). Only two ketones were detected and GLC analyses indicated that these compounds were more polar than the saturated isomeric ketone standards. Bromination verified that these ketones were unsaturated and an IR spectral scan of one of the ketones is shown in Figure 1. A scan of a saturated ketone is included for reference. Bands at 1645, 990 and 910 cm^{-1} indicated the presence of a terminal double bond and Table V presents carbon skeleton-hydrogenolytic cleavage data which again give carbon number and position of the carbonyl groups. The two ω- unsaturated ketones were therefore identified as tetradec-13-en-2-one and tetradec-13-en-4-one.

Alcohols were identified by similar procedures with some of the data shown in Table VI. For example, retention times showed these alcohols to be more polar than the saturated secondary alcohol tetradecan-3-ol and, carbon skeleton data again gave chain length and position of the hydroxyl group. Further, IR analyses verified the terminal double bond, all of which led to the identification of these alcohols as tetradec-13-en-3-ol and -4-ol.

Other microorganisms possessing the ability to oxidize methyl groups of alkanes may also be capable of oxidizing the terminal double bond of alk-1-enes (10). Since it appeared that the terminal double bond was left intact during the subterminal oxidation of tetradec-1-ene by *Penicillium*, no further studies were conducted on the oxidation of the alk-1-ene.

To determine if the saturated isomeric alcohols and their corresponding ketones simply represented end products of an incomplete oxidative pathway, the saturated alcohols and

TABLE IV

Carbon Skeleton Analyses of Ketones from Tetradecane

Ketones	Carbon fragments[a]
Standard	
Tetradecan-4-one	14 and 10
Tetradecan-3-one	14 and 11
Tetradecan-2-one	14 and 12
Experimental	
Tetradecan-4-one	14 and 10
Tetradecan-3-one	14 and 11
Tetradecan-2-one	14 and 12

[a]See Table II.

TABLE V

Carbon Skeleton Analyses of Unsaturated Ketones From Tetradec-1-ene

Ketones	Carbon fragments[a]
Standard	
Tetradecan-2-one	14 and 12
Experimental	
Tetradec-13-en-2-one	14 and 12
Tetradec-13-en-4-one	14 and 10

[a]See Table II.

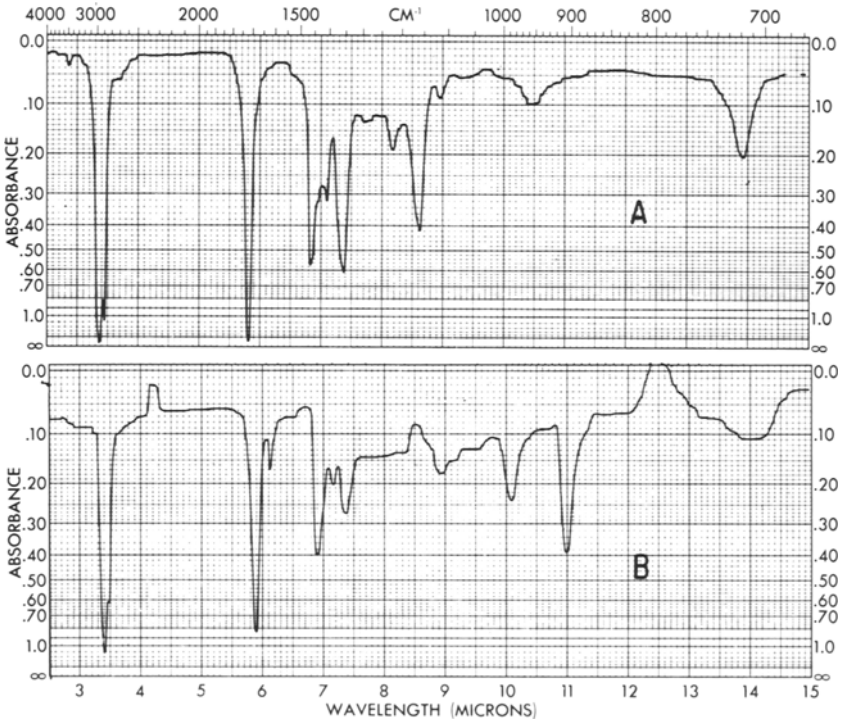


FIG. 1. IR absorption spectra of (A) standard tetradecan-2-one and (B) experimental unsaturated ketone from the oxidation of tetradec-1-ene by *Penicillium.*

ketones were supplied as sole carbon sources for growth of the organism. All three isomers of the two compounds supported growth.

Hopkins and Chibnall in 1932 (11) suspected that ketones were involved in paraffin oxidation by fungi when they observed that *Aspergillus versicolor* grew at the expense of long chain paraffins as well as ketones of the corresponding chain length. Ketones and secondary alcohols have been implicated as intermediates with other microorganisms capable of oxidizing aliphatic hydrocarbons. Lukins and Foster (12) showed that acetone, butan-2-one, pentan-2-one and hexan-2-one were produced from the respective *n*-alkanes by *Mycobacterium smegmatis*, and Fredricks (13) identified decan-2- 3-, 4- and 5-one, together with the corresponding secondary alcohols, from the oxidation of decane by *Pseudomonas aeruginosa*. An *Arthrobacter* species was shown by Klein and coworkers (14,15) to cooxidatively transform several *n*-alkanes to the respective 2, 3 and 4 ketones and corresponding secondary alcohols. These alkanes would not serve as sole carbon and energy sources, and the ketones formed were not further metabolized. Recently, Vestal and Perry (16), using a *Brevibacterium* species in isotope competition experiments, recovered acetone as an intermediate in propane oxidation.

TABLE VI

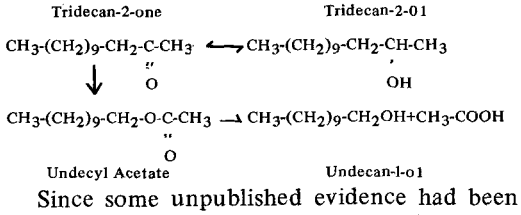
Alcohols From Tetradec-1-ene

	Retention time, min[a]	Carbon skelton fragments[b]
Standard		
Tetradecan-3-ol	14.0	14 and 11
Experimental		
Tetradec-13-en-3-ol	16.9	14 and 11
Tetradec-13-en-4-ol	16.2	14 and 10

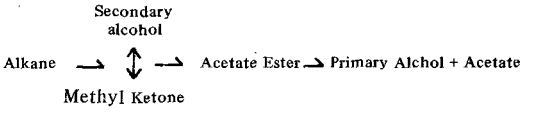
[a]GLC analyses were performed at 180 C using an 8 ft column with 5% FFAP as the liquid phase.

[b]See Table II.

At this point it is pertinent to mention some work which at its inception appeared to be a digression from hydrocarbon oxidation. A study was initiated in our laboratory on the oxidation of the C_{13} methyl ketone, tridecan-2-one, by *Pseudomonas multivorans* isolated from an enrichment culture on the methyl ketone. During oxidation of this ketone the C_{11} primary alcohol, undecan-1-ol, accumulated until substrate depletion, whereupon the undecan-1-ol was oxidized. It was proposed that the C_{13} methyl ketone was split to the C_{11} alcohol plus a C_2 fragment (17). Subsequently, an intermediate was isolated which would account for these results (undecyl acetate, which is cleaved by the organism to undecan-1-ol and acetate). The secondary alcohol, tridecan-2-ol, was also formed from the ketone and if this alcohol was supplied as substrate, the ketone, acetate ester and primary alcohol were formed (18). *P. aeruginosa* also carried out these reactions and this organism was used for preliminary cell-free enzymatic studies with tridecan-2-one-3-^{14}C (19). Unfractionated extracts, in the presence of oxygen and NADH or NADPH formed radioactive undecyl acetate. Hydrolysis of the recovered radioactive ester yielded labeled undecan-1-ol (Table VII). When the labeled ester, undecyl acetate-2-^{14}C was employed with unfractionated extracts, labeled acetate was detected. These data led us to propose the following degradative pathway for tridecan-2-one:



Since some unpublished evidence had been obtained which indicated that tetradecan-2-ol was formed from oxidation of the C_{14} alkane by this organism, we grew *P. aeruginosa* on the C_{13} alkane and looked for oxidation products by GLC analysis (20). Two alcohols were detected: tridecan-2-ol and undecan-1-ol. It is evident from our previous statements that, if the same subterminal catabolic pathway was operative, it would generate a secondary alcohol or ketone of substrate chain length and a primary alcohol shorter by two carbons. Although the ketone and acetate ester were not apparent in the above analysis, no further attempts were made to detect them since the carbon numbers and structures of the alcohols identified fulfill the precursor-product requirements of the pathway which is presented in a generalized form as follows:



Some preliminary experiments have been completed with cell-free extracts from *Penicillium* in attempts to determine if the alkane was metabolized via the pathway visualized for subterminal alkane oxidation by *Pseudomonas*. The fungus was grown on the alkane, and acetone powders were prepared and extracted with phosphate buffer plus Lubrol. Cell extract from

TABLE VII

TLC and Radioactivity Data on Ester Produced From Tridecan-2-one-3^{14}C by Cell-Free Extract of *P aeruginosa*

Compounds	R_f[a]	DPM
Authentic undecyl acetate	.70	
Experimental ester	.70	47,555
Experimental after hydrolysis	.20	1,515
Authentic undecan-1-ol	.21	

[a]Solvent system for TLC was hexane-ether-acetic acid (85:15:2 v/v).

TABLE VIII

Identification of Ester Intermediate From Tridecan-2-one-3^{14}C Oxidation by a Cell-Free Preparation From *Penicillium*

	Retention time	DPM
Standard undecyl acetate	5.6[a]	---
Experimental ester	5.7	1875
TLC alcohol spot after ester hydrolysis	---	1700
Preparation GLC of alcohol spot	5.1[b]	445
Trimethylsilyl derivative	6.6	---
Standard undecan-1-ol	4.5	---
undecan-1-ol-TMS	6.6	---

[a]GLC analyses employed a 15 ft, 10% FFAP column at 175 C.
[b]GLC analyses employed an 8 ft, 6% Apiezon L column at 160 C.

TABLE IX

Alcohols From C_{13} and C_{14} Acetate Esters[a]

	Retention time, min	Trimethylsilyl derivative
Standard undecan-1-ol	5.0	6.0
Standard dodecan-1-ol	8.3	9.9
From undecyl acetate substrate experimental undecan-1-ol	5.3	6.2
From dodecyl acetate substrate experimental dodecan-1-ol	8.6	9.9

[a]GLC analyses, see Table VIII.

cells grown on tridecane were incubated with tridecan-2-one-3-^{14}C plus NADH to determine if the ester, undecyl acetate, was produced. Diisopropylfluorophosphate was added to the reaction mixture in an attempt to inhibit hydrolysis of postulated ester. In Table VIII it can be seen that a radioactive intermediate was produced with a retention time comparable to undecyl acetate. Hydrolysis, after addition of cold carrier undecyl acetate, yielded a radioactive primary alcohol as determined by TLC. The fraction from the TLC plate was eluted and recovered by preparative GLC. Retention times showed it to be radioactive undecan-1-ol.

Cell-free extracts were then checked for esterase activity. Extracts prepared from cells grown on tridecane and tetradecane were incubated with the respective acetate ester, i.e., undecyl acetate and dodecyl acetate. Reaction mixtures were analyzed for the primary alcohol resulting from ester hydrolysis (Table IX). Extracts from cells grown on tridecane exhibited exterase activity on undecyl acetate as seen by the formation of undecan-1-ol and, correspondingly, extracts from tetradecane grown cells hydrolyzed dodecyl acetate forming dodecan-1-ol. When these esters were supplied to an extract from cells grown on glucose no esterase activity was seen.

With the identification of the secondary alcohol, methyl ketone and acetate ester in the *Penicillium* system, it appears that a subterminal pathway for hydrocarbon oxidation, initiated at the methylene α to the methyl group, is occurring.

Since it has been shown above that the 3- and 4-methylene groups of the hydrocarbon are also oxidized and, that the resulting oxidation products (3- and 4-alcohols and ketones) supported growth, the interesting question remains, i.e., are all the ketonic isomers oxidized via ester intermediates?

ACKNOWLEDGMENT

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Hydrocarbon Cooxidation in Microbial Systems[1]

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ABSTRACT

This review summarizes the present status of hydrocarbon cooxidation in microorganisms. Hydrocarbons, which cannot be used for growth by many soil microorganisms, can be oxidized if present as co-substrates in systems in which another substrate is furnished for growth. Paraffinic, cycloparaffinic and aromatic hydrocarbon cooxidations have been demonstrated. Most hydrocarbon cooxidation reactions seem to involve the incorporation of molecular oxygen by mono- and dioxygenases. From paraffinic hydrocarbons, products accumulating in fermentation systems include acids, alcohols, aldehydes and ketones. Usually, the initial attack is at the terminal methyl group in paraffin oxidations. The only products isolated in the cycloparaffins have been ketones. Extensive studies have been carried out on cooxidation of mono- and dicyclic aromatic hydrocarbons. Oxidation of methyl substituents on aromatic rings usually results in the accumulation of the aromatic mono acid or alcohol. Dihydroxylation of the aromatic ring has been observed. Products of aromatic ring rupture arise via both ortho and meta cleavage pathways.

INTRODUCTION

It is the purpose of this paper to present the current status of microbial cooxidation of hydrocarbons which leads to the accumulation of products. I shall apply the term cooxidation to those systems containing essentially two components, one, a hydrocarbon that cannot, or only partially, be used for growth, and the second, a growth substrate, which may or may not be a hydrocarbon. This could just as well be termed a review of biotransformations of hydrocarbons which would perhaps be more familiar to those in the steroid field. In the field of fermentation chemistry, particularly steroids, the concept of one-step changes or transformations of substrate molecules is now commonplace.

As originally defined by Foster (1), cooxidation was a system in which "nongrowth hydrocarbons are oxidized when present as cosubstrates in a medium in which one or more different hydrocarbons are furnished for growth." I would like to expand on this concept because over the years we have found that several hydrocarbons can be transformed where the growth substrate is a nonhydrocarbon. Also it is not surprising that some microorganisms have been found to grow on a particular hydrocarbon without significant product accumulation, other than cells. Upon introduction into a multisubstrate system only partial oxidation of the hydrocarbon occurs with a resulting build up of product. Recently a term, cometabolism, has been introduced into the literature (2) to describe systems in which compounds very similar in structure to the substrate to be oxidized are used as growth substrates. I regard these systems as another form of cooxidation or transformation. There appears to be increasing evidence that cooxidation could be quite widespread in nature.

Cooxidation of hydrocarbons can be divided into roughly two categories; incorporation of molecular oxygen via the monooxygenases into paraffinic structures, and oxidation of aromatic rings of hydrocarbons by the dioxygenases. However, this division and the participation of molecular oxygen has not been rigorously proven by enzyme isolation and ^{18}O studies.

PARAFFINIC HYDROCARBON COOXIDATION

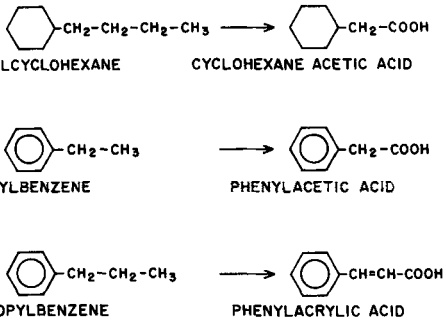
Leadbetter and Foster (3) reported that *Pseudomonas methanica*, growing on methane, oxidized ethane, a nongrowth substrate, to acetic acid, ethanol and acetaldehyde.

$$C_2H_6 \xrightarrow[CH_4]{\text{P. METHANICA}} CH_3COOH, C_2H_5OH, CH_3CHO$$

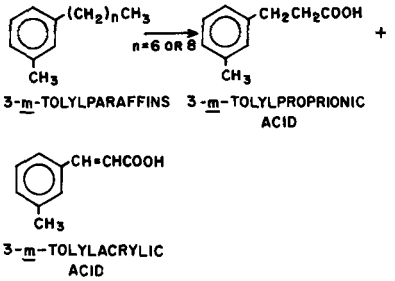
Similar products resulted from propane and butane oxidation except that the respective ketone, acetone and 2-butanone were also detected. Molecular oxygen was implicated by ^{18}O studies. Yields of products were very low, usually less than 1 g/liter, but this is not unexpected in a closed gas system.

Davis and Raymond (4) observed that strains of *Nocardia*, *Mycobacterium* and *P. aeruginosa* carried out the following transformations while growing on *n*-alkanes or an alkyl moiety:

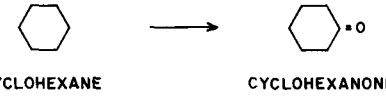
[1]One of five papers being published from the Symposium "Biochemistry of Hydrocarbon Degradation," presented at the AOCS Meeting, Chicago, September 1970.



Under the conditions tested (four liter stirred fermenters), yields of ~1.0 g/liter were obtained. Douros and Frankenfeld (5) reported that under conditions of cooxidation *P. ligustri, P. pseudomaleii, P. orvilla, Alcaligenes sp, Cellulomonas galba* and *Brevibacterium healii* transformed *n*-amylbenzene to *trans*-cinnamic acid. Hexadecane was the growth substrate, and yields in skaken flasks were of the order of 5g/liter. Slow utilization of the alkylbenzene for growth was noted in the absence of hexadecane. These authors (6) also found that a strain of *Micrococcus cerificans* carried out oxidation of 3-*m*-tolyl paraffinics.



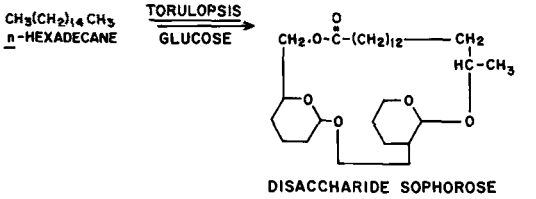
A typical reaction for cycloparaffins as reported by Ooyama and Foster (7) is shown below:



In this instance 2-methylbutane was the growth substrate for a gram negative rod. Concentrations of the ketones were of the order of 0.1-0.3 g/liter.

Further evidence of cooxidation of paraffinic hydrocarbons conforming to Foster's definition is lacking. However, in a number of studies, paraffinic hydrocarbons have been transformed in good yields when nonhydrocarbon growth substrates have been used.

Tulloch et al. (8), working with *Torulopsis apicola* isolated from sow thistle petals, found that the yield of an extra-cellular glycolipid could be increased several-fold when even-numbered hydrocarbons from C_{16} to C_{24} were used. In the presence of high concentrations of glucose the alkanes were oxidized to ω- and ω-1-hydroxy acids and subsequently incorporated into novel crystalline sophorsides. A greatly abbreviated example follows:



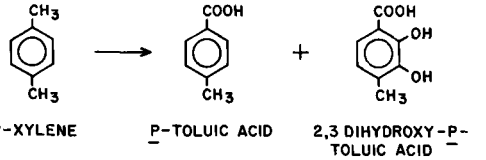
Yields were better than 10 g/liter. Subsequently, Jones and Howe (9) and Jones (10), using *Torulopsis gropengiesseri*, reported similar transformations for alkanes, alk-1-enes and branched-chain alkanes. Transformation of *n*-hexadecane at concentrations of 20 g/liter in 5-liter stainless steel fermenters was almost 100%

Klein et al. (11) found that when an *Arthrobacter* strain, isolated from soil and selected for slow utilization of hexadecane as a sole-C-source, was grown in a hexadecane- or pentadecane-salts medium supplemented with yeast extract or corn steep liquor, mono hexadecanones or pentadecanones accumulated.

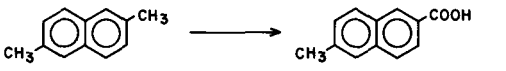
The three and four ketones were also present but in lesser amounts. Conversion of the alkanes to ketones was about 2 1/2%, with ketone concentration reaching 2.0 g/liter after 120 hr of incubation in shaken flasks.

AROMATIC HYDROCARBON COOXIDATION

In our laboratory we have carried out extensive studies on the oxidation of aromatic hydrocarbons in cooxidation systems. In 1967, we reported the oxidation of mono- and dicyclic aromatic hydrocarbons by several soil isolates of the genus *Nocardia* (12). Strains of *N. corallina, N. salmonicolor,* and *N. minima* were shown to oxidize *p*-xylene to 2,3- dihydroxy-*p*-toluic acid and *p*-toluic acid.



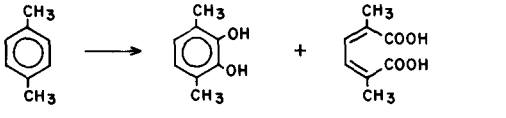
Oxidation of a methyl group on the naphthalene ring was possible, but dihydroxylation of these dicyclic aromatics was not observed.



2,6-DIMETHYLNAPHTHALENE 6-METHYL-2-NAPHTHOIC ACID

In these studies, the growth substrate was *n*-hexadecane and product accumulation was found to be highly dependent upon pH and concentration of both the growth and non-growth substrates. It might be noted that control of these factors was difficult at the shaken flask level, and that eventually we developed a system for studying cooxidation which employed weakly basic anion exchange resins (13). Use of resins in agar plates has permitted screening of many more systems than was possible by shaken flasks, and results are much more reproducible and reliable.

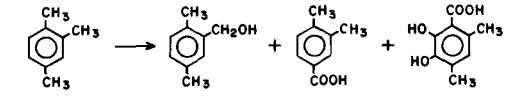
The first evidence for ring-splitting of aromatics under cooxidaton conditions was reported by Jamison et al. (14). They found that a new strain of *N. corallina* V-49, accumulated α,α'-dimethyl-*cis,cis*-muconic acid as the cooxidation product of *p*-xylene. The dimethylcatechol was isolated and shown to be a possible intermediate in the following reaction:



P-XYLENE 3,6-DIMETHYLPYRO-CATECHOL α,α'-DIMETHYL-CIS,CIS-MUCONIC ACID

Hosler and Eltz (15) scaled up this system in a pilot-plant fermenter with an operating volume of 520 liters. By continuous feed of the growth substrate (*n*-paraffins, lard oil or Cerelose) and control of xylene at 3-20 mg/liter with a hydrocarbon analyzer on the exhaust gas, they were able to achieve a muconic acid concentration of 20 g/liter in a 40 to 50 hr fermentation period. The optimal pH for this transformation was 6.5 to 7.0. Using chloramphenicol to stop protein synthesis, they were able to demonstrate that the oxygenases were inducible.

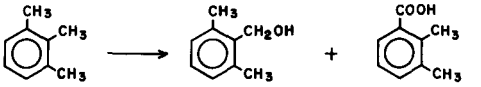
In addition to *p*-xylene cooxidation, *N. corallina* V-49 has also been shown to oxidize a fairly wide array of aromatics. Pseudocumene (1,2,4-trimethylbenzene) is oxidized to a number of products.



PSEUDOCUMENE 2,5-DIMETHYLBENZL ALCOHOL 3,4-DIMETHYL BENZOIC ACID 2,3-DIHYDROXY-4,6-DIMETHYL BENZOIC ACID

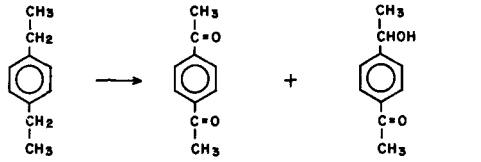
Traces of what we think is trimethylmuconic acid have also been isolated in this reaction.

In a study of all the other methyl substituted monocyclic aromatic hydrocarbons (16), 1,2,3-trimethylbenzene yielded products as shown:



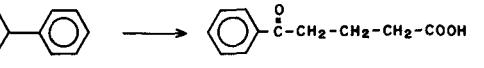
1,2,3-TRIMETHYLBENZENE 2,6-DIMETHYL BENZYL ALCOHOL 2,3-DIMETHYL BENZOIC ACID

The only other product was the mono acid (2,3,4-trimethylbenzoic acid) from 1,2,3,4-tetramethylbenzene. When the meta and para diethylbenzenes were tested they gave unexpected results, as demonstrated for the para diethylbenzene below:



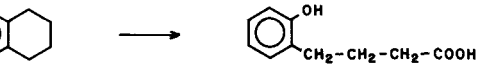
P-DIETHYLBENZENE P-DIACETYLBENZENE 4-(1-HYDROXYETHYL)-ACETOPHENONE

The effect of still larger substituents on the monocyclic aromatics have not been fully assessed, but we have identified 4-benzoylbutyric acid as a product of biphenyl cooxidation.



BIPHENYL 4-BENZOYL-BUTYRIC ACID

Another mechanism of oxidation was indicated when the unsubstituted naphthalenes were investigated. Of the three tested, naphthalene, tetralin and indane, only tetralin was oxidized.



TETRALIN 4-PHENYL-(2'-HYDROXY)-BUTYRIC ACID

This was a surprising result since we had previously investigated many cycloparaffinic structures in cooxidation systems without finding any evidence of oxidation.

After examining the ten possible dimethyl naphthalene isomers with this *Nocardia* it appears that oxidation occurs at a methyl substituent on the 2,7-, 2,6-, 1,2- and 1,7-dimethylnaphthalenes. For this culture we have concluded that the area shown under the dotted line in the structure below must be unsubstituted to get transformation:

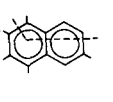


TABLE I

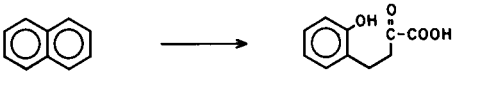
Preliminary Screen on Resin Plates

Hydrocarbon[a]	*Nocardia sp.* 87-5	*N. coeliaca* 95-5	*Streptomyces sp.* 97-14
Benzene	---	2.0[b]	---
Toluene	0	2.0	0
P-xylene	2.3	0.4	4.0
Biphenyl	5.6	---	6.2
Decalin	1.0	1.5	0
Tetralin	2.3	---	0.8
Naphthalene	91.0	58.0	72.3

[a]Growth substrate, *n*-hexadecane.
[b]mg/plate

In addition to these compounds, several unsubstituted and methyl-substituted three-ring aromatic hydrocarbons have been studied, but no indication of oxidation was noted.

A group of soil isolates with quite different oxidation patterns from the *N. corallina* types is represented by *N. coeliaca* 95-5, *N. species* 87-5 and *Streptomyces* sp. 97-14. Preliminary screening data obtained on resin agar plates with *n*-hexadecane as the growth substrate are shown in Table I. All three strains can grow slowly on naphthalene as the only source of carbon, but product accumulation is observed when *n*-hexadecane or Cerelose is the growth substrate. The naphthalene product was identified as 4-(2-hydroxyphenyl)-2-ketobutyric acid, a new product. The complete work of Davies and Evans (17) on the oxidation of naphthalene by a pseudomonad gave no indication of this product. We have confirmed, by isolation of α-naphthol which arises nonenzymatically from



NAPHTHALENE — 4-(2-HYDROXYPHENYL)-2-KETOBUTYRIC ACID

naphthalenediol, that the initial oxidative steps are similar to the *Pseudomonas* pathway. Methyl substituted naphthalenes were not as easily oxidized, but ring-splitting occurred if one ring was unsubstituted. The oxidation of biphenyl was found to yield 4-benzoylbutyric acid. It is interesting that the two *Nocardia* cultures also have a limited ability to oxidize *p*-xylene to dimethylmuconic acid, indicating that both the ortho and meta cleavage pathways are operable.

TABLE II

Preliminary Screen on Resin Plates of *Achromobacter cycloclastes*

Hydrocarbon[a]	mg/plate
Benzene	44.0
Toluene	4.8
P-xylene	1.5
Naphthalene	37.7

[a]Growth substrate, *n*-hexadecane.

Other microorganisms which we have investigated for transformations include *Achromobacter cycloclastes* and a *Corynebacterium*. The *Achromobacter* was found to accumulate products from several hydrocarbons as shown in Table II. The benzene and naphthalene products were identified as phenol and gentisic acid, respectively. In the case of the *Corynebacterium*, Hill (18) found that although naphthalene and anthracene were oxidized in the absence of a cosubstrate to salicyclic and 2-hydroxy-3-naphthoic acids, respectively, the use of Cerelose as a growth substrate increased the concentration of these acids manyfold.

In conclusion, this review has shown that many soil microorganisms possess the capability of transforming a wide variety of hydrocarbon substrates. Under conditions of cooxidation oxygenated products are accumulated from hydrocarbons which cannot serve as growth substrates. In addition, there is growing evidence that product accumulation may result from several hydrocarbons, both paraffinic and aromatic, which serve as growth substrates if a second substrate is present for growth. We also believe that in many of the soil microorganisms, more than one pathway of aromatic hydroxylation and ring-splitting may be present. Consequently, it is not possible to generalize about the most prevalent pathway for the breakdown of aromatic hydrocarbons in nature at this time.

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The Effect of Undernutrition on the Development of Myelin in the Rat Central Nervous System

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ABSTRACT

Newborn rats were segregated into litters of 6 and 22 pups. Undernourished groups were maintained for 21 and 53 days at which time a portion of each group was sacrificed and the remainder refed until 121 days. The amount of myelin isolated from the central nervous system was decreased in nutritionally deprived animals but there was little change in its chemical composition or interference in the normal chemical maturation of the membrane. The only consistent change was a reduction in phosphatidyl ethanolamine plasmalogen. This deficit tended to be corrected by nutritional rehabilitation and was most complete in those animals refed ad lib. diets for the longest time periods prior to sacrifice. The relatively normal chemical composition of myelin obtained from undernourished animals differs from changes described in myelin membranes isolated from patients with destructive or degenerative diseases of the nervous system. This seems to be further evidence that the reduction in myelination in undernutrition is a result of decreased synthesis of the membrane.

Undernutrition during early development adversely affects the growth of the brain in humans and in experimental animals (1-11). Suckling rats with restricted food intake exhibit a reduction in brain weight and in the content of DNA, RNA, protein (7) and lipids (1-5,9) in whole brain. Phospholipids and cholesterol are reduced to 75-80% of that found in normal littermates (3,5). However, glycolipids (3,9), proteolipid proteins (5) and plasmalogens (4), lipid classes considered to be closely associated with the myelin membrane, exhibit greater deficits in undernourished animals and are only 50% to 70% of control values. Corroboration of decreased myelin deposition in the brains of malnourished rats was provided by Benton et al. (5), who demonstrated myelin to be decreased on histologic sections stained by a modified Loyez method. Thus, the deleterious effects of undernutrition on myelinogenesis have been demonstrated both biochemically and histologically.

The chemical composition of myelin, isolated by ultracentrifugation, from undernourished animals has not been described, and it is not known whether despite a large reduction in the lipid content of the brains of nutritionally deprived animals the chemical composition of the isolated membrane undergoes the same maturational changes seen in siblings (12-18). The present investigation demonstrates that the chemical composition of isolated myelin during maturation is, with minor exceptions, similar in severely undernourished animals and their normally fed littermates, even though the undernourished animals exhibit a 25% reduction in brain weight and a 30% reduction in the amount of myelin isolated from their brains when compared to normally fed siblings.

MATERIALS AND METHODS

Preparation of Samples

Sprague-Dawley rats obtained from Simonsen Laboratories, White Bear, Minn., were segregated within 12 hr after birth into litters of 6 and 22 pups. When weaned at 21 days, undernourished animals were fed balanced diets to maintain their body weights at approximately 25% of that of control siblings under 53 days of age. Groups of undernourished animals were placed on unrestricted balanced diets at 21 and 53 days of age. At 21, 53 and 121 days after birth, animals were sacrificed by the intrathoracic injection of sodium pentobarbital and their brains rapidly removed omitting the olfactory lobes. The brains were weighed and immediately homogenized in 0.32 M sucrose. Myelin was separated by the method of Suzuki et al. (19). The isolated membrane was evaluated by electron microscopy; it was more than 95% pure.

Estimation of Lipid and Protein Composition of Isolated Myelin

The isolated membrane was lypholized, weighed and an aliquot used for the determination of protein by the method of Lowry et al. (20). The remainder was extracted by the method of Folch et al. (21). The total lipid

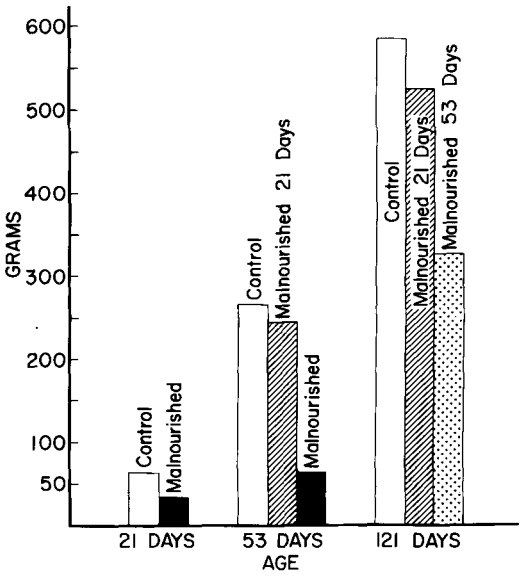

FIG. 1. Effect of malnutrition and subsequent re-feeding on the body weights of rats.

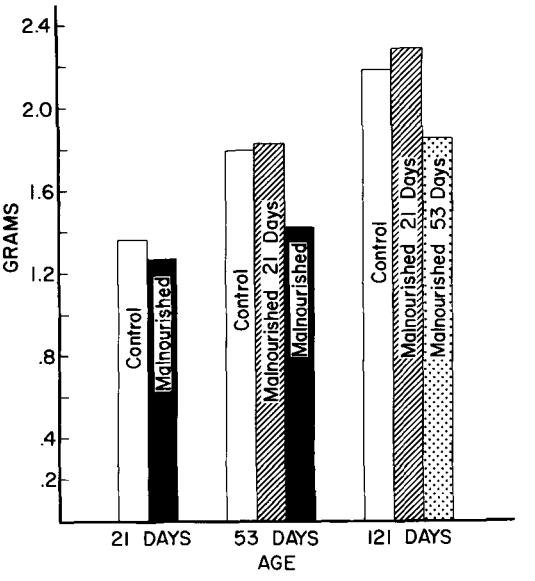

FIG. 2. Effect of malnutrition and subsequent re-feeding on the brain weights of rats.

content was determined by weighing an aliquot of the lower phase. The phosphorous content of the lower phase was estimated by the method of Rouser et al. (22), the proteolipid protein content by the method of Lowry et al. (20) as modified by Hess and Lewin (23), the cholesterol content by the method of Zlatkis et al. (24), the glycolipid content by the method of Hess and Lewin (23). Individual phospholipid classes were estimated after separation by two dimensional thin layer chromatography (TLC) as described by Rouser et al. (25). To estimate the plasmalogen content of ethanolamine, the lipids were separated by TLC by the method of Yanagihara and Cumings (26).

Determination of Fatty Acids

Individual phospholipids were isolated by TLC as described above, visualized with water, spots removed and, in the presence of the silica gel, hydrolyzed and methylated in 5% methanolic-HCl in a sealed tube at 65 C for 18 hr. In the case of phosphatidyl ethanolamine, the dimethylacetals were separated from the fatty acid methyl esters by the method of O'Brien et al. (27) prior to gas liquid chromatography (GLC).

Glycolipids were isolated by silicic acid column chromatography according to the method of Rouser et al. (28). Cerebrosides and sulfatides were then separated by one dimensional TLC on 0.25 mm Silica Gel G plates with a developing solvent of chloroform-methanol-water (130:40:4 v/v). The compounds were visualized with water, removed and hydrolyzed in 8% methanolic HCl at 95 C for 18 hr. The hydroxy and nonhydroxy fatty acids were then separated by one-dimensional TLC on 0.25 mm Silica Gel G plates, using hexane-ether (8:3 v/v) as the developing solvent.

Fatty acids were estimated on a Barber-Coleman, Model 5000 Gas Chromatograph equipped with a flame detector. Peaks were identified by the use of reference standards and by logarithmic plots of retention time versus chain length and degree of saturation. Helium was used as the carrier gas. Fatty acids derived from phospholipids were separated on a 6 ft, 7% DEGS columns at 170 C. Nonhydroxy fatty acids derived from cerebroside and sulfatide were analyzed on 6 ft, SE 30 columns, programmed at 2 C/min from 170 C to 205 C; while hydroxy fatty acids were analyzed on SE 30 columns programmed at 2 C/min from 185 C to 205 C after being converted to their trimethyl silyl derivatives. Fatty acid standards were obtained from Applied Science Laboratories, State College, Pa. All solvents were redistilled prior to use.

RESULTS

The average body weights of rats undernourished from birth to 21 days and from birth to 53 days were 52% and 24% of the values of their respective controls (Fig. 1). The brains of 21-day undernourished animals weighed 93% of those of normally fed siblings while at 53 days

TABLE I

Effect of Undernutrition on the Composition of Rat Central Nervous System Myelin

Age of rats in days at time of killing	Total protein, % dry weight	Expressed as per cent total lipid				Expressed as per cent recoverable phospholipid				
		Proteolipid protein	Cholesterol	Glycolipid	Phospholipid	Sphingomyelin	Phosphatidyl serine + inositol	Phosphatidyl choline	Phosphatidyl ethanolamine	Plasmalogen[a]
21 Days										
Control	31.4	20.0	22.7	19.6	41.0	6.4	14.4	35.0	44.2	61.5
Undernourished	28.7	17.1	22.4	20.5	38.1	6.9	13.8	33.4	46.1	55.6
53 Days										
Control	28.8	23.0	19.0	23.6	33.5	8.5	16.5	28.8	46.1	74.3
Undernourished	29.2	26.6	21.0	24.2	35.4	9.9	17.1	28.8	44.1	51.5
Undernourished for 21 days	27.5	26.4	22.2	25.4	34.9	9.4	19.9	27.8	43.0	68.8
121 Days										
Control	29.8	28.1	21.0	28.6	34.7	10.1	16.9	26.0	47.0	65.7
Undernourished for 53 days	31.8	24.4	21.4	26.0	33.1	6.5	16.5	28.8	48.3	57.2
Undernourished for 21 days	30.1	23.6	21.6	27.0	32.9	7.5	18.7	29.5	44.5	61.0

[a]Expressed as per cent of total phosphatidyl ethanolamine.

the average brain weight of undernourished animals was 78% of controls (Fig. 2). In those animals who were refed after 21 days of malnutrition and then sacrificed at 53 days, the body weights had returned to 92% of control values while their brain weights did not differ from controls. When similar animals were sacrificed at 121 days, neither the body nor the brain weights differed from controls; however, the brain weights of those animals that had been starved for 53 days and then sacrificed at 121 days had returned to only 85% of control values. The weight of the myelin recovered from 21-day-old undernourished animals was 86.5% of that amount isolated from a similar number of controls while in animals underfed for 53 days it was reduced to 71% of control values.

Lipids accounted for 80% to 85% of the dry weight of the myelin membrane in all groups of animals studied. However, the proportion of the various lipid classes found in the myelin membrane did vary with age (Table I). Glycolipids increased from 19.6% of the total lipid at 21 days of age to 28.6% at 121 days of age, and a similar increase was noted in the proportion of total lipid accounted for by proteolipid protein. Concurrently, the total phospholipid concentration decreased from 41% at 21 days to 34.7% at 121 days. Much of this decrease in the percentage of phospholipid could be accounted for by the drop in phosphatidyl choline which at 21 days was 35% and at 121 days was 26% of the total recoverable phospholipid. Sphingomyelin increased from 6.4% to 10.1% of the recoverable phospholipid during this period.

Maturational changes in the composition of fatty acids of various lipid subclasses are noted on Tables II, III and IV. Maturation was associated with a decrease in the proportion of C18:0 and increase in the proportion of C 18:1 fatty acids present in phosphatidyl ethanolamine and phosphatidyl choline. There were no consistent changes in the fatty acid composition of sphingomyelin (Table II). The main changes seen in the fatty acid composition of cerebrosides and sulfatides during maturation consisted in a decrease in the relative amount of intermediate chain length compounds (C 18 through C 22) and an increase in the relative amount of longer chain length fatty acids (C 23 and C 24). The greatest change noted in these glycolipids was the increase in the percentage of nervonic acid (C 24:1) (Tables III and IV).

With few exceptions, the myelin membrane of the undernourished animals showed the same maturational changes as their control siblings (Table I). The major change in lipid class composition was a decrease in the plasmalogen form

TABLE II

Effect of Undernutrition on the Composition of Phospholipid Fatty Acids in Rat Central Nervous System Myelin[a]

Individual fatty acids	Sphingomyelin					Phosphatidyl ethanolamine					Phosphatidyl choline				
	21 Day		53 Day		121 Day	21 Day		53 Day		121 Day	21 Day		53 Day		121 Day
	C[b]	U	C	U	C	C	U	C	U	C	C	U	C	U	C
14:0	2.4	4.6	2.5	1.2	1.5	—	—	—	—	—	—	—	—	—	—
16:0	11.4	20.4	8.0	8.0	13.7	10.0	7.1	8.7	13.6	13.4	38.0	40.6	34.0	30.2	36.1
16:1	1.0	0.7	—	1.6	0.8	1.1	1.0	0.9	3.1	1.5	Trace	Trace	Trace	0.5	Trace
18:0	31.5	29.8	23.1	22.7	27.4	27.8	29.2	15.4	14.5	13.4	31.2	30.4	29.9	25.4	23.1
18:1	8.4	8.8	2.2	7.7	8.5	42.1	44.1	50.0	52.3	54.0	20.4	21.1	27.0	35.2	32.7
20:0	3.3	5.5	6.5	4.9	2.0	2.4	1.8	1.7	1.0	0.9	2.1	1.4	1.2	1.1	0.8
20:1	3.3	10.9	4.2	4.2	2.3	6.9	7.5	12.0	7.9	9.7	1.5	1.2	1.8	2.0	1.9
20:2	—	—	—	—	—	1.3	1.0	1.6	0.8	1.2	Trace	0.7	0.5	Trace	Trace
20:3	—	—	—	—	—	Trace	Trace	1.6	1.3	1.0	—	—	Trace	Trace	—
20:4	—	—	—	—	—	1.2	1.0	1.7	1.0	1.2	1.8	0.9	0.5	1.0	0.5
22:0	6.9	2.8	6.4	6.1	4.6	4.7	4.9	3.3	3.0	1.8	2.2	2.4	1.7	2.0	3.4
22:1	3.0	1.5	7.1	4.4	7.0	Trace	Trace	Trace	Trace	0.1	Trace	0.7	2.1	1.0	Trace
22:2	—	—	—	—	—	Trace	—	1.0	0.3	Trace	1.2	Trace	0.4	—	Trace
24:0	8.2	9.3	12.9	9.4	8.8	2.5	2.0	2.0	1.1	2.1	2.1	1.5	1.8	1.9	1.9
24:1	19.7	5.7	26.7	29.6	23.2	Trace	Trace	Trace	Trace	0.4	—	—	—	Trace	Trace

[a]Each fatty acid expressed as per cent of total fatty acids.
[b]C, control; U, undernourished.

TABLE III

The Effect of Undernutrition on the Composition of Glycolipid Nonhydroxy Fatty Acids in Rat Central Nervous System Myelin[a]

Individual fatty acid	Sulfatides					Cerebrosides				
	21 Day		53 Day		121 Day	21 Day		53 Day		121 Day
	C[b]	U	C	U	C	C	U	C	U	C
14:0	0.8	Trace	1.0	0.3	0.8	—	—	0.6	Trace	0.9
16:0	9.3	4.7	14.6	7.6	4.6	2.9	2.5	3.8	1.8	1.8
18:0	26.9	22.2	20.8	30.9	14.9	14.0	12.7	8.4	9.1	5.0
18:1	Trace	Trace	Trace	11.6	1.1	—	—	Trace	0.6	Trace
20:0	4.3	5.6	5.2	2.6	2.6	4.7	4.5	3.8	3.7	2.6
20:1	0.1	Trace	0.5	1.1	0.8	Trace	Trace	0.1	0.2	0.1
22:0	13.5	15.8	11.5	6.5	8.5	18.0	18.4	12.7	10.4	8.5
22:1	Trace	Trace	0.7	0.8	1.7	1.3	2.5	0.9	1.4	1.8
23:0	2.5	2.4	2.1	1.2	3.2	2.7	2.5	3.8	2.7	4.2
24:0	42.0	48.5	37.8	22.2	38.2	48.0	42.0	39.0	31.4	30.4
24:1	1.3	0.9	6.2	13.9	30.2	9.1	13.4	17.9	31.0	39.1
25:0	—	—	—	—	—	Trace	Trace	4.5	2.3	2.5
25:1	—	—	—	—	—	—	—	1.2	0.6	0.8
26:0	—	—	—	—	—	Trace	0.1	3.0	2.3	1.9
26:1	—	—	—	—	—	Trace	Trace	Trace	Trace	Trace

[a]Each fatty acid expressed as per cent of total fatty acids.
[b]C, control; U, undernourished.

of phosphatidyl ethanolamine, which after 53 days of undernutrition was only 70% of that found in control animals though the total phosphatidyl ethanolamine values were comparable. The only consistent change found in the fatty acid composition of lipid classes was an increase in the proportion of C 24h:1 and C 24:1 in cerebrosides (Table III and IV).

Feeding undernourished animals unlimited amounts of a balanced diet tended to partially correct the decrease in plasmalogens. Those animals refed after 21 days of deprivation and sacrificed at 121 days had plasmalogen values that were 93% of control values while those refed after 53 days of undernutrition had plasmalogen values that were only 87% of control levels at 121 days (Table I). The composition of cerebroside fatty acids in 121-day-old animals undernourished for 21 days closely resembled their controls. However those animals underfed for 53 days continued to demonstrate an increase in the proportion of C 24h:1 and C 24:1 in cerebrosides when sacrificed at 121 days (Tables III and IV).

DISCUSSION

Changes in the lipid composition of myelin during maturation have been described previously. It is generally agreed that glycolipids increase as a proportion of the total lipid while phospholipids, particularly phosphatidyl choline, decrease with age (12-18). Eng and Noble (14) also noted an increase in ethanolamine glycerophosphate plasmalogen. There is less certainty about the concentration of cholesterol in the developing myelin membrane. In the highly purified preparations of Eng and Noble, there was a relatively insignificant change in cholesterol concentration with age. Dalal and Einstein (17) found a significant increase in the mole percentage of cholesterol during development. However, as much as 40% of the lipid in myelin prepared from their younger animals was not characterized. The changes noted in the lipid composition of isolated myelin obtained from our control rats examined at 21, 53 and 121 days are in general agreement with those reported in the literature (12-14,16).

Maturational changes in the fatty acid composition of the individual lipid classes in the myelin membrane have also been reported. O'Brien and Sampson (29) noted that during human maturation the realtive amount of C 18:0 fatty acids decreased while C 18:1 increased in both phosphatidyl choline and phosphatidyl ethanolamine. These findings were not corroborated by Skrbic and Cumings (30) working with rat myelin from 21 days to adult life; they found no difference in the fatty acid composition of phosphatidyl choline during this period. Our findings agree closely with those of O'Brien and Sampson, since we found that the proportion of C 18:0 fatty acid in phosphatidyl ethanolamine decreased 50% and in phosphatidyl choline decreased 25% from 21 to 121 days. The relative increase of C 18:1 fatty acid in phosphatidyl ethanolamine was 30% and in phosphatidyl choline was 60%

TABLE IV

Effect of Undernutrition on the Composition of Glycolipid Hydroxy Fatty Acids in Rat Central Nervous System Myelin[a]

Individual fatty acid	Sulfatides					Cerebrosides				
	21 Day		53 Day		121 Day	21 Day		53 Day		121 Day
	C[b]	U	C	U	C	C	U	C	U	C
18h:0	7.3	6.2	3.3	3.6	1.5	1.2	0.8	0.7	0.8	0.4
18h:1	Trace	0.1	0.1	0.1	0.5	Trace	—	—	0.1	0.2
20h:0	Trace	0.6	0.8	1.1	Trace	1.4	2.4	1.0	1.0	1.9
22h:0	26.6	28.6	23.4	25.0	20.2	28.6	27.4	22.4	21.2	16.2
22h:1	—	—	—	—	—	—	—	—	0.4	Trace
23h:0	4.2	3.2	6.6	6.2	10.0	4.9	4.5	9.4	7.6	10.9
23h:1	—	—	—	—	—	Trace	Trace	Trace	Trace	Trace
24h:0	61.4	61.2	66.0	59.5	64.0	60.5	57.5	59.0	50.5	51.0
24h:1	—	—	Trace	4.5	4.2	3.4	7.3	7.4	15.4	16.7
25h:0	—	—	—	—	—	—	—	0.6	1.6	2.1

[a]Each fatty acid expressed as per cent of total fatty acids.
[b]C, control; U, undernourished.

during this period. Prior reports indicate that there is an increase in the C 24:1 content of cerebroside and sulfatide during maturation (29,31); our studies supports these findings.

The meaning of changes in the lipid class concentration of isolated myelin during maturation is uncertain. As early as 1966, Davison et al. (12) suggested that myelin isolated early in life might consist of two membranes, one resembling more mature myelin and the other resembling glial-plasma membrane. Recently, work by Agrawal et al. (18) has confirmed this hypothesis demonstrating two components of myelin isolated early in life. One membrane contained only trace amounts of cerebrosides and large amounts of phospholipids and cholesterol, while the second membrane contained considerable cerebroside and much less phospholipid, particularly phosphatidyl choline. They suggested that when initially formed, myelin resembles the glial-plasma membrane and with maturation is converted into compact myelin concomitant with an increase in cerebrosides and a decrease in phosphatidyl choline. Our studies of myelin isolated from severely malnourished rats at 21 and 53 days of life suggest that this membrane is capable of undergoing the same maturational changes as occur in normally fed siblings. The single exception is the reduction of the plasmalogen form of phosphatidyl ethanolamine in the membrane isolated from poorly nourished animals. This exception may occur because the biosynthetic pathway for plasmalogens differs to some degree from that described for other phospholipids. The recent work of Schmid and Takahaski (32) suggests that alcohols rather than fatty acids are utilized for plasmalogen biosynthesis. Culley (33) noted that the ratios of C 16:0 to C 18:0 and C 18:0 to C 18:1 ester-bound fatty acids were increased and the ratio of C 24:1 to C 24:0 fatty acids in glycolipids was decreased in the brains of rats malnourished for 24 and 50 days. We were unable to find similar changes in analyzing the fatty acid composition of individual lipid class obtained from isolated myelin.

This study is the first to demonstrate directly that significantly less myelin can be isolated from the brains of undernourished animals than can be isolated from the brains of normally fed controls. Earlier studies of the amount of myelin present in the brains of undernourished animals have been inferential and based on analysis of whole brain lipids. Dobbing (1) demonstrated a 30% reduction in brain cholesterol of nutritionally deprived suckling rats; Culley and Mertz (3) showed that in undernourished animals the concentrations of cholesterol and phospholipid were reduced to 70-75% of control values while the concentration of cerebroside was more severely effected, only 55% of control values. Plasmalogens were found to be reduced to a greater degree than other phospholipids by Culley et al. (4). Similar results were found by Benton et al. (5) who also demonstrated a 30% reduction in proteolipid proteins. Chase et al. (8) found that the incorporation of ^{35}S sulfate into the lipid fraction of brains from poorly nourished animals was reduced approximately 50% when compared to controls.

Myelin has now been isolated in a number of neurologic disorders, including Niemann-Pick's Disease (34), spongy degeneration (35), subacute sclerosing leukoencephalitis (36),

Schilder's disease (37), and generalized gangliosidosis (38). Changes in the chemical composition of the membrane in these disorders have been similar though of varying magnitude. Generally, cholesterol has been elevated and ethanolamine phosphatides, glycolipids and proteolipid proteins reduced. Analyses of the fatty acid content of major lipid classes have been performed on several occasions and indicate that there are reductions in the concentration of longer chain components. These alterations in the chemical composition of the membrane have been considered nonspecific (34). O'Brien and Sampson (39) found that there was a specific defect in the chemical composition of myelin obtained from patients with metachromatic leukodystrophy. The membrane contained excessive amounts of sulfatide and was deficient in cerebroside. All of these diseases can be classed as degenerative or destructive diseases of the nervous system in which myelin is either directly attacked or is destroyed secondarily as a result of destruction of nerve cells or their axons. None of these changes are noted in myelin isolated from undernourished animals; this appears to be further evidence that the reduction in myelination in undernutrition is the result of a decrease in the synthesis of the membrane.

The chemical data thus far obtained do not explain why myelin synthesis is significantly reduced in undernourished suckling animals. Considerably less DNA is found in the brains of severely undernourished animals and it is possible that undernutrition has a direct affect on the multiplication of the oliogodendroglial cells. Another possibility is that there is a significant reduction of the neuronal population of the cerebral hemispheres prior to myelination though histological evidence of significant neuronal loss at this point is lacking (40,41). A third possibility is that in the central as well as in the peripheral nervous system, the number of myelin lamellae surrounding an axon is directly proportional to its diameter (42) and that in undernourished animals axonal growth during maturation is curtailed though the axon itself is not destroyed. This could result in fewer lamellae about each axon and less myelin in the brains of starved animals. Support of any of these hypothesis will require further correlation of biochemical and quantitative histological data.

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Ether-Containing Lipids of the Slime Mold, *Physarum polycephalum*: I. Characterization and Quantification

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ABSTRACT

Rapidly growing plasmodia of the acellular slime mold, *Physarum polycephalum*, contain large amounts of ether-linked lipids. The ether bonds occur principally in the phospholipids, where plasmalogens account for 21-24 mole per cent of the total and alkyl ether phospholipids comprise 12 mole per cent of the total. Plasmalogens account for over half of the ethanolamine phosphatides, while the alkyl ether derivatives are more uniformly distributed among the various phospholipid fractions. The 16 carbon side chain is by far the major component of both the saturated and the α,β-unsaturated ether derivatives. The nature and amounts of ether lipids present in *Physarum* add to the growing evidence that Myxomycetes are more closely related to protozoa than to fungi.

INTRODUCTION

Within recent years, representative species of almost every phylum have been examined to determine the types of structural lipids present. It is therefore surprising that so little study has been devoted to the Phylum Myxophyta, the slime molds. We have had occasion to examine plasmodia of the acellular slime mold, *Physarum polycephalum*, for ether-linked lipids. The abundance and variety of ether lipids found may be of considerable taxonomic importance in relating myxomycetes to other phyla.

MATERIALS AND METHODS

Culture Conditions

The isolate of *Physarum polycephalum* used in this study was obtained from C.J. Alexopoulos, Department of Botany, University of Texas. The plasmodium developed in laboratory culture from spores obtained from a cross of isolates from Costa Rica and Jamaica.

Stock cultures of micro-plasmodia were maintained as follows: initially, sterile, cotton-plugged narrow-mouthed 500 ml Erlenmeyer flasks containing 100 ml of liquid growth medium (1) were inoculated with axenic plasmodia migrating on nutrient agar (1). For innoculation, 1 in. squares of plasmodia on agar were used. The culture flasks were rotated in the dark at 25 C on a New Brunswick model S-3 gyratory shaker at 120 rpm. After liquid shake cultures were established, subsequent stock cultures were maintained by transferring 2 ml of densely settled micro-plasmodia to fresh media. Uniform inoculation of flasks to be used for sampling could be achieved using this procedure. The rapid growth rate of micro-plasmodia necessitates reinoculation every 72 hr. The micro-plasmodia to be used for analysis were collected daily and stored at -34 C. Before freezing, the culture medium was decanted from the plasmodia, which rapidly settle to the bottom of the flasks.

Harvesting and Extraction of Lipids

When required for extraction, the plasmodia were thawed, care being taken not to allow the temperature to exceed 4 C. Traces of the medium were removed by resuspending twice in cold distilled water and centrifuging at 1090 x *g* for 10 min below 4 C in a Sorvall RC-2B centrifuge. The plasmodia (generally from 3-30 g fresh weight) were homogenized for 2 min in a Waring blendor in 3 vol. of chloroform-methanol 1:2 according to the Bligh and Dyer method (2). The plasmodia were assumed to contain greater than 95% water, and the volume of solvent mixture used for extraction was adjusted accordingly. The residue after filtration through sintered glass was reextracted with chloroform-methanol 1:1, and chloroform was added to make a final chloroform-methanol ratio of 2:1. This extract was then washed with 0.1 M KCl according to the procedure of Folch et al. (3). The completeness of extraction was determined by refluxing a sample of the re-extracted residue with 6 N HCl for 16 hr and then measuring the fatty acids liberated by gravimetric analysis after extraction into diethyl ether.

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TABLE I

Analysis of Pooled Chromatographic Fractions of Physarum

Fraction	Chloroform-methanol ratio of eluting solvent	Phosphorus, μg atoms	Aldehyde,[a] μmoles	Glyceryl alkyl ethers, μmoles	Principal components detected after acid hydrolysis
1	9:1 (fractions 1-9)	7.9	2.3	1.5	Unidentified sugar
2	6:1 (fractions 18-29)	22.3	11.7	2.9	Ethanolamine
3	1:1 (fractions 36-42)	10.5	0.63	2.1	Serine, inositol, lysine or β-alanine
4	1:1 (fractions 43-57)	27.1	0.13	1.9	Choline
5	1:9 (fractions 58-72)	2.6	<0.1	Not examined	Choline

[a]The aldehyde values shown above were measured by the fuchsin reaction. These results were confirmed by a second column chromatography.

Analytical Procedures

Phosphorus was measured by the method of Bartlett (4) modified by digestion with perchloric acid (5). Phosphorus present on thin layer plates was determined by the method of Rouser et al. (6) modified by using Silica Gel H (Merck) plates instead of a silica gel plain-magnesium silicate mixture, and by an increase in digestion time from 20 to 50 min. Aldehydogenic lipids were assayed either by condensation of released aldehyde with the fuchsin reagent (7) or by reaction of the α-β unsaturated ether linkage with iodine (8) using palmitaldehyde dimethylacetal and cetyl vinyl ether, respectively, as standards. 1-0-Glyceryl ethers were isolated by the method of Hanahan and Watts (9) as modified by Thompson and Lee (10), and were assayed as described by Thompson and Kapoulas (11). Isopropylidene derivatives of the glyceryl ethers were prepared by the method of Hanahan et al. (12). Dimethylacetals were obtained by hydrolysis of intact lipids with methanolic-HCl (13).

Column chromatography was carried out using silicic acid (Mallinckrodt) previously washed with methanol and ether, and activated at 140 C for 16 hr.

Column Chromatography

Samples of the total lipid extract were chromatographed on columns (approximately 1 g silicic acid per 5 mg lipid) packed in chloroform and maintained at 4 C to reduce the possibility of plasmalogen degradation. Fractions of approximately 10 ml were collected and monitored by thin layer chromatography (TLC). Neutral lipid was eluted with chloroform (150 ml). The following solvent mixtures were then passed through the column: chloroform-methanol, 9:1 (150 ml); chloroform-methanol, 6:1 (140 ml); chloroform-methanol, 1:1 (200 ml); and chloroform-methanol, 1:9 (200 ml). The recovery of phosphorus and aldehyde from the column was approximately 90%.

Based on TLC analysis in solvents 1 and 2, various eluates were divided into five major fractions. Aliquots of the fractions were examined for phosphorus and plasmalogen content, and, after a 3 hr hydrolysis with 2 N aqueous HCl at 100 C, for base content by paper chromatography and electrophoresis.

TLC was carried out on either Silica Gel G or H plates (Merck). The following solvent systems were used for resolving the various phospholipids 1. chloroform-methanol-acetic acid-water, 65:43:3:1 (14; 2. chloroform-methanol-concentrated ammonia, 70:30:5; and 3. chloroform-methanol-acetic acid-water, 50:25:8:4 (15). Ascending paper chromatography was carried out on Whatman No. 1 paper in butanol-acetic acid-water, 4:2:1 or propanol-water, 80:20. Amino groups were detected with ninhydrin, choline with Dragendorff or phosphomolybdic acid reagents, and inositol as described by Battaglia et al. (16).

Gas liquid chromatography of fatty aldehyde dimethylacetals and isopropylidene derivatives of 1-0-glyceryl ethers was carried out on a Barber-Colman Series 5000 chromatograph equipped with a flame ionization detector. A 6 ft glass column of 15% polyethylene glycol succinate on Anakrom AB, 60-70 mesh, was used for the analysis of both derivatives. A column temperature of 140 C and a nitrogen pressure of 12 lb/sq in. was employed for the separation of dimethylacetals, while a column temperature of 173 C and a pressure of 18 lb/sq in. was used for isopropylidene derivatives.

RESULTS

Rate of Plasmodial Growth

The plasmodial growth rate during the loga-

rithmic phase was determined by following the increase of dry weight over a period of 24 hr as follows: 24 hours after inoculating a fresh culture flask with micro-plasmodia, 2 ml aliquots were taken in duplicate with sterile Pasteur pipettes approximately every 6 hr over a 24 hr period. The plasmodia were collected on weighed 19 mm millipore discs, flushed twice with distilled water, dried overnight in a vacuum oven at 40 C and weighed. The data showed that there was a doubling of dry weight approximately every 6 hr during the sampling period.

Lipid Content

At least 95% of the lipid present in the tissue was recovered under the extraction conditions described. The lipid content of the growing plasmodium is 3.83 mg/g fresh weight or 68.6 mg/g dry weight. Lipid analysis revealed the presence of 63 μmoles lipid phosphorus, 14.8 μmoles aldehydogenic lipid, and 9.2 mg neutral lipid per gram dry weight.

The figure for neutral lipid present represents the amount of lipid eluted from a silicic acid column with chloroform. This fraction, which contained approximately 5% of the total aldehydogenic material, was found by TLC to consist mainly of free fatty acids, sterols and lesser amounts of triglycerides.

A sample of total lipid corresponding to 81 μmoles phosphorus and 18 μmoles aldehyde was chromatographed on a silicic acid column. From the data shown in Table I, it is clear that most of the aldehydogenic activity was associated with the second of the five fractions collected. This fraction appeared to be at least 90% ethanolamine phosphoglycerides by TLC analysis and by analysis of water soluble hydrolysis products. The aldehyde-phosphorus ratio of this fraction of 0.52:1 compared very well with the α,β-unsaturated ether-phosphorus ratio of 0.55:1. The other main aldehyde-containing component occurred in much smaller amounts than ethanolamine plasmalogen and was present in Fraction 1 along with two other compounds as judged by TLC. This substance moved a little beyond the origin in the ammoniacal TLC solvent system but towards the solvent front when chromatographed in acidic solvents. Fifty per cent of the phosphorus present in Fraction 1 was recovered in the same area of thin layer chromatograms as the aldehyde-containing substance. This principal component had the same Rf as authentic phosphatidic acid in the solvent system chloroform-methanol-acetone-acetic acid-water, 50:10:20:10:5 (17).

Fractions 1, 2, 3 and 4 all contained substantial amounts of saturated ether linkages. Approximately 65% of the ethanolamine phosphoglyceride fraction is present as one or the other form of ether. Although the choline phosphoglyceride fraction (Fraction 4) contained appreciable amounts of saturated ethers, it was virtually devoid of aldehydogenic linkages.

All fractions contained traces of pigments, but the bulk of these substances were eluted with Fractions 1 and 3. The compounds have not been characterized.

Analysis of the Ether Side Chain Distribution

By far the most prevalent side chain occurring in both the saturated and unsaturated ether linkages examined is 16 carbon atoms long. Ninety and 85% by weight of the saturated ether groups in the ethanolamine and choline phosphoglyceride fractions, respectively, are present as chimyl alcohol. Both fractions contain small amounts of 16:1 side chains (7.5% and 5%, respectively) but only trace amounts of other components, including 18:1 and 18:0 were observed. More than 97% by weight of the α,β-unsaturated ether side chain in the ethanolamine phospholipid fraction is recovered as palmitaldehyde after hydrolysis.

DISCUSSION

The plasmodia of *Physarum polycephalum* have a remarkably high content of ether-containing lipids. Plasmalogens account for more than 20 mole per cent of the total phospholipids, and one of the two major fractions, the ethanolamine phosphatides, contains over 50 mole per cent of the plasmalogen analog. *Physarum* therefore must go on record as having one of the highest concentrations of plasmalogens yet reported. By comparison, some mammalian brain and skeletal muscle phospholipids, among the richest sources known, contain as much as 27 mole per cent and 22 mole per cent of plasmalogens, respectively (18).

The level of alkyl ether phospholipids is approximately 12 mole per cent of the total lipid phosphorus—again, unusually high. Altogether, the two types of ether lipids account for 35% of all phospholipid molecules.

The large amounts of ethers and the overwhelming predominance of the C_{16} side chain are reminiscent of the patterns found in certain protozoa. *Tetrahymena pyriformis*, while containing no plasmalogens, has an abundance of alkyl ether lipids, which consist almost exclusively of the $C_{16:0}$ analog (19). Other protozoa have been reported to contain plasma-

logens, particularly in their ethanolamine lipids (20,21).

The similarities between lipids of *Physarum* and those of the protozoa may be important from a taxonomic point of view. There is disagreement as to whether *Physarum* and the other true slime molds should be classified among the protozoa or fungi (22). Although the somatic phase is animal-like, particularly in the case of the swarm cells, the reproductive structures are more plant-like in structure. The fact that ether lipids have not been found in fungi might imply that these organisms are less closely related to the slime molds than are the protozoa. This conclusion is in agreement with that reached by Korn et al. (23), who found that the unsaturated fatty acid pattern of *Physarum* strongly resembles that of *Acanthamoeba...*

Two properties of *Physarum*, its rapid growth rate and its high content of ether lipids, recommend it as a potentially useful system for studying ether lipid metabolism. Part II of this work describes our initial efforts in this direction.

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Ether-Containing Lipids of the Slime Mold, *Physarum polycephalum*: II. Rates of Biosynthesis

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ABSTRACT

The in vivo incorporation of 1-^{14}C-palmitic acid and 1-0-[8,9-^{3}H] hexadecyl glycerol (chimyl alcohol) by plasmodia of the slime mold, *Physarum polycephalum* has been studied. ^{14}C-palmitate rapidly enters ester and alkyl ether side chains of phospholipids, but alk-1-enyl side chains are labeled more slowly. ^{3}H-chimyl alcohol is incorporated into the alkyl ether phospholipids, which appear to undergo enzymatic desaturation, producing plasmalogens. The feasibility of *Physarum* as the source of a cell-free enzyme system for plasmalogen synthesis is discussed.

INTRODUCTION

Lipids of the myxomycete *Physarum polycephalum* are exceptionally rich in plasmalogens and alkyl ether phosphatides (1). We have followed the incorporation of radioactive palmitic acid and chimyl alcohol into *Physarum* plasmodia. The appearance in *Physarum* plasmalogens of significant amounts of radioactivity after relatively short time intervals establishes its value for studying plasmalogen biogenesis.

MATERIALS AND METHODS

Physarum polycephalum plasmodia were grown and extracted as described in the accompanying paper (1). All labeling experiments were carried out in the logarithmic phase of growth. 1-^{14}C-palmitic acid (54 mc/mmole) was supplied by the New England Nuclear Corporation. The preparation of 1-0-[8,9-^{3}H] hexadecyl glycerol (1 x 10^3 mc/mmole) has been described previously (2).

Incubation Conditions

The labeled precursors, dissolved in 0.2 ml of absolute ethanol, were transferred with sterile Pasteur pipettes to culture flasks containing 2 to 3 g wet weight of plasmodia in 100 ml of medium. The flasks were then incubated in the dark as discussed in the companion paper (1). Incubations containing the labeled fatty acid were removed from the shaker after 3 hr, and the plasmodia were allowed to settle. The medium was then decanted, and the shaking was continued after the addition of 100 ml of fresh medium. This procedure was carried out to eliminate the continued uptake of fatty acid during the incubation period. Owing to the much slower uptake of chimyl alcohol, incubations containing this substance were not treated in a similar fashion, but were shaken continuously without the removal of nonincorporated label. At the end of the incubation period, each flask was frozen quickly in acetone-dry ice after decanting most of the medium, and then stored at -20 C until analysis could be made by procedures described in the accompanying paper (1).

Lipid Analyses

1-0-Alkyl glyceryl ethers and 1-0-alk-1-enyl glyceryl ethers were prepared by hydrolysis with lithium aluminum hydride as described by Albro and Dittmer (4), but with one modification. The ether-soluble residue remaining after hydrolysis was dissolved in diethyl ether and then washed with water. This washing was found to be necessary to remove small amounts of salts which affected the resolution of the alkyl and alk-1-enyl glyceryl ethers on Silica Gel G plates in petroleum ether-diethyl ether-glacial acetic acid 30:70:1 (by volume).

The variability of the yield of radioactivity recovered from lithium aluminum hydride reaction mixtures (80-92%) prompted the use of an alternative method for the assay of the alk-1-enyl ether group. This method was based on the conversion of the aldehydogenic lipids to the corresponding dimethylacetal derivatives by reaction with a methanol/HCl mixture, and subsequent separation of these compounds from fatty acid methyl esters and from compounds containing the saturated ether group by thin layer chromatography (TLC) on Silica Gel G plates in xylene. The methanolysis was carried out as described by Farquhar (5) but without saponification of the hydrolysis products.

In some instances the aldehydogenic group was determined by the two-dimensional chromatographic technique of Horrocks (6). The sample was chromatographed on the left-hand

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TABLE I

Incorporation of ^{14}C-Palmitic Acid Into Alkyl and Alk-1-enyl Glyceryl Ethers[a]

Incubation period, hrs	Alkyl glyceryl ethers	Alk-1-enyl glyceryl ethers	Fatty acids remaining free after incubation
3	6.75	0.87	56.5
6	7.9	1.95	64.8
12	15.8	3.3	26.4
24	14.0	10.5	22.6
30	13.25	9.1	16.3
48	8.45	11.45	5.9

[a]Per cent of radioactivity associated with plasmodia after harvest.

edge of a 10 x 20 cm Silica Gel G plate in chloroform-methanol-concentrated NH_4OH (70:30:5), and after fuming with HCl, the plate was developed in the second dimension with petroleum ether-diethyl ether-glacial acetic acid (70:30:1). Aldehyde zones could be detected by spraying the plate with 2,4-dinitrophenylhydrazine reagent.

Phospholipid separations by TLC were conducted as described (1). The solvent system chloroform-methanol-concentrated NH_4OH (70:30:5) tended to be the more useful, although at times any free fatty acid moved only slightly above the phosphatidyl ethanolamine area. To eliminate the possibility of contamination of one zone with the other, the plate was generally redeveloped in petroleum ether-diethyl ether-glacial acetic acid (30:70:1). Neutral lipids were generally resolved by chromatography on Silica Gel G plates in petroleum ether-diethyl ether-glacial acetic acid (70:30:1).

Preparative TLC was carried out as follows: Pooled samples (1/10) representative of each incubation period were chromatographed on 20 x 20 cm Silica Gel G plates, previously washed with chloroform-methanol-water (60:35:8), using the chloroform-methanol-concentrated NH_4OH solvent system. After redevelopment in petroleum ether-diethyl ether-glacial acetic acid (30:70:1), the three major zones were eluted with 30 ml of either chloroform-methanol 1:4 or, in the case of the unknown zone which moved near the origin, absolute methanol. The yield of radioactivity recovered from ethanolamine and choline phosphoglycerides, and from the neutral lipid area was in excess of 90%.

Radioactivity Measurements

Radioactive samples were assayed in a Packard Tri-Carb liquid scintillation spectrometer with efficiencies of 49% for ^{3}H and 89% for ^{14}C. The method of counting ^{3}H and ^{14}C bound to silica gel plates has been described previously (8). Quenching was determined by means of an external standard.

Lipids were viewed by exposure of plates to iodine vapor. Aldehydogenic substances were located by spraying the plates with 2,4-dinitrophenylhydrazine reagent.

RESULTS

Uptake of ^{14}C-Palmitic Acid Into Phospholipids

In experiments where 1-^{14}C-palmitic acid (11 x 10^6 dpm) was added to logarithmic phase cultures of *Physarum*, approximately 30% of the radioactivity was associated with the plasmodia when they were harvested after a 3 hr incubation. Following replacement of the ^{14}C-palmitate-containing medium by fresh, unlabeled medium, incorporation of the plasmodia-associated radioactivity into phospholipids proceeded fairly rapidly, ranging up to 67% after 24 hr. There was no further increase in incorporation into phospholipids during an additional 24 hr, although incorporation did continue into compounds which appeared to be triglycerides on the basis of TLC mobility.

As expected, a sizable proportion of the radioactivity incorporated into phospholipids by the plasmodia was recovered in ether-linked side chains, Analysis of the ether derivatives after $LiAlH_4$ reduction revealed that the alkyl ethers accumulated a maximum of radioactivity by 12 hr and then declined, while the alk-1-enyl ethers gained radioactivity more slowly but continuously through the entire 48 hr period surveyed (Table I). The radioactivity not accounted for in the Table existed in the form of esterified fatty acids.

The identity of the alkyl glyceryl ether was confirmed by isolating the labeled material by preparative TLC, converting it to the isopropylidene derivative, and examining the product by preparative gas liquid chromatography (GLC). Eighty-nine per cent of the radioactivity had the same retention time as an authentic isopropylidene of chimyl alcohol. Greater than 95% of the radioactivity of the gas-chromatographic eluate had the same mobility on TLC

TABLE II

Incorporation of ^{3}H-Chimyl Alcohol Into Ether and Ester Derivatives

Per cent of total plasmodia-associated radioactivity recovered	Incubation time, hr			
	1	3	6	9
As unreacted ^{3}H-chimyl alcohol	68[a]	70	48	22
Covalently bound in tissue lipids	32	30	52	78
Per cent of covalently bound lipids recovered present as				
1-0-Alkyl Ethers	59	48	50	43
1-0-Alk-1-enyl Ethers	16	16	23	32
Esterified fatty acids	10	8	6	12

[a]All figures represent the mean values from duplicate incubations. The duplicate values varied by less than 12%.

(petroleum ether-diethyl ether-glacial acetic acid 70:30:1) as authentic isopropylidene derivatives.

The identity of the alk-1-enyl group was confirmed by preparing dimethylacetal derivatives of the incubation mixtures and, after saponification of fatty acid methyl esters, isolating the dimethylacetals by GLC. More than 95% of the radioactivity recovered from the column had the same retention time as the dimenthylacetal of palmitaldehyde.

It is not clear why plasmodia isolated in the in vivo incubation experiments retain such a large percentage of free fatty acid. That portion not yet incorporated into complex lipids by the end of 3 hr requires much additional time to be utilized. This fraction of the fatty acid may be unavailable to the metabolic system of the plasmodia by virtue of being either adsorbed to the plasmodial cell wall or sequestered into some type of intracellular vacuole.

Evidence favoring this interpretation was obtained by the following experiment. Plasmodia (5.3 g wet weight), incubated with labeled palmitic acid for 24 hr, were homogenized as described by Brewer et al. (9). Aliquots of the 600 x *g* (15 min) supernatant were incubated at 37 C with a series of reagents, including 2 mM ATP, 5 mM magnesium chloride, 2 mM cytidine triphosphate (CTP), 1 mM NAD, 1 mM NADP and 5 μg Triton X-100/ml. The free fatty acids associated with the plasmodia (58% of total label at zero time) were quickly incorporated into phospholipids. After 30, 60 and 120 min, the respective free fatty acid levels were 18%, 8% and 6%. The explanation for the fatty acids' unavailability in the intact organism will require further study.

Uptake of ^{3}H-Chimyl Alcohol

^{3}H-Chimyl alcohol (4.13 x 10^{6} counts/min) was incubated with plasmodia for four different time intervals (1, 3, 6 and 9 hr), each experiment being carried out in duplicate. The chimyl alcohol was absorbed more slowly than palmitate, and less then 25% of the radioactive substrate was taken up at any of the time intervals studied.

TLC in the neutral lipid solvent mixture showed that, apart from unincorporated chimyl alcohol, the major radioactive lipid components remained at the origin, suggesting that they were of phospholipid nature. The percentage of the total plasmodia-associated radioactivity incorporated into this area increased from 21% after 1 hr to 53% after 9 hr. Aliquots of the incubation extracts were reacted with methanolic HCl. The data shown in Table II

TABLE III

Per Cent Incorporation of ^{3}H-Chimyl Alcohol Into Phospholipids[a]

Phospholipid area	1 hr	3 hr	6 hr	9 hr
Phosphatidyl choline	2.7	2.7	4.7	6.6
Phosphatidyl ethanolamine	5.4	4.3	12.7	17.6
Unknown	14.3	8.9	12.9	24.9

[a]The figures shown above represent the mean values of per cent of the plasmodia-associated radioactivity that was incorporated in duplicate incubations. The per cent incorporation into the various fractions of duplicate incubations varied by less than 10%.

TABLE IV

Incorporation of ^{3}H-Chimyl Alcohol Into Alkyl and Alk-1-enyl Ether and Ester Linkages of Ethanolamine Phosphoglycerides[a]

Incubation period, hr	Alkyl ether	Ester	Alk-1-enyl ether
1	46.1	17.9	30.7
3	35.5	19.7	38.5
6	26.9	14.3	53.8
9	17.9	18.5	59.1

[a]Per cent of total radioactivity.

demonstrate that most of the radioactivity was present in ether linkage, confirming that there had been little degradation of the saturated ether bond. These results also support the hypothesis that the alk-1-enyl ether is formed from the saturated ether by some form of dehydrogenation process. This is especially indicated by the 3 and 6 hr figures since, although there is little change in the per cent incorporation into ester linkages, the decrease in per cent incorporation into the saturated ether linkage quite closely parallels the increased incorporation into the unsaturated ether moieties.

The distribution of radioactivity among individual lipid fractions was examined by TLC of the total lipid extracts in the ammoniacal phospholipid solvent. Apart from neutral lipid, three major radioactive zones were detected. Two of the radioactive compounds were found in the phosphatidyl choline and phosphatidyl ethanolamine areas, respectively, of thin layer chromatograms while the other substance remained near the origin in this solvent system, but moved near the solvent front in most acidic solvents. The per cent incorporation into each fraction was found to increase with time (Table III).

Localization of Radioactive Unsaturated Ethers

Most of the α,β-unsaturated ether groups present in lipid extracts of the organism are found in the ethanolamine phosphoglyceride fraction (1). It therefore seemed likely that the bulk of the labeled dimethylacetals liberated after reaction with methanolic HCl was being derived from this fraction. To confirm this the three major phospholipid zones were each isolated by preparative TLC and treated with methanolic HCl. The results of the analyses of ethanolamine phosphoglycerides are shown in Table IV. In this fraction the decrease in radioactivity present as saturated ethers closely matches the increase in the per cent incorporation into the unsaturated ethers.

No clear-cut trend was discernible in the choline phosphoglyceride fraction. In this case, acyl esters accounted for 45-65% of the total radioactivity while alkyl ethers and dimethylacetals contained 24-41% and 4-8% of the ^{3}H. respectively. No more than 2% of the radioactivity in the neutral lipid fraction was associated with acyl esters or alk-1-enyl ethers.

The recovery of radioactivity from the unknown phospholipid area was below 50% in all cases where lipid was eluted from chromatographic plates. Therefore, the per cent incorporation into aldehydogenic linkages of this lipid was determined by the two-dimensional TLC technique of Horrocks (6), in which plasmalogens chromatographed in one dimension are cleaved with HCl to free aldehydes, which are then developed in the second dimension.

The percentages of the total plasmodia-associated radioactivity present in the unknown lipid fraction as alk-1-enyl ether side chains was 1.6%, 1.9%, 5.1% and 14.4% at 1, 3, 6 and 9 hr, respectively. A comparison of these results with the percentages reported in Table III for the unknown lipid indicates that at 9 hr over half the radioactivity in this fraction is in the form of plasmalogens.

In Vitro Incorporation Studies. In addition to the incorporation of ^{14}C-palmitate by cell free preparations described above a few preliminary studies have been made with other substrates. Incubation of a 275 Xg supernatant with ^{14}C-cetyl alcohol in the presence of 5 μg Triton X-100 per milliliter and ATP, CoA, Mg Cl_2 NADPH and glyceraldehyde-3-phosphate, essentially as described by Kapoulas and Thompson (10), failed to produce significant incorporation into lipids. However, ^{3}H-chimyl alcohol incubated with a 120 Xg (15 min) supernatant under similar conditions for 2 hr at 35 C showed a conversion of approximately 20% of the radioactivity into a polar lipid. Unlike the polar lipid found in vivo, this product remains at the origin of thin layer plates chromatographed in acidic or basic solvent systems. Characterization of this material has not yet been attempted.

DISCUSSION

The origin of the ether-linked side chain of

plasmalogens has been the subject of much speculation and frustrated experimental effort (11). Conflicting findings still prevent general agreement on the pathway of plasmalogen formation.

Working with the terrestrial slug *Arion ater*, Thompson obtained evidence suggesting that plasmalogens arise through the enzymatic desaturation of alkyl ether phospholipids (12). Later studies showed that slugs fed alkyl ethers labeled in the side chain with ^{14}C and in the glyceryl moiety with ^{3}H synthesized plasmalogens having a $^{3}H/^{14}C$ ratio similar to that of the administered substrate (13). The same precursor-product relationship seems to exist in tissues as diverse as rat brain (14), dogfish liver (15), and ascites tumor cells (16,17).

On the other hand, Ellingboe and Karnovsky (18), using the digestive gland of the starfish, *Asterias forbesi*, found fatty aldehydes to be more efficient than fatty alcohols as precursors of alk-1-enyl ethers while the reverse was true in the formation of alkyl ethers. The authors concluded that no direct precursor-product relationship exists between the two ether types.

A serious handicap in studies of plasmalogen biosynthesis is the sluggish rates at which administered substrates enter these lipids. Most in vivo experimental systems require many hours before there is sufficient incorporation for analysis. The development of useful cell-free preparations from such tissues would seem most unlikely. We feel that our experience with *Physarum* demonstrates it to be not only an excellent organism for in vivo studies but, more importantly, a promising source of active cell-free enzymes.

The uptake of ^{14}C-palmitate into alkyl ethers is rapid, reaching a maximum after 12 hr. As with all other tissues studied, the alk-1-enyl ethers show a much slower incorporation of label, but the incorporation continues long after the bulk of added palmitate is esterified into lipids. By 48 hr, the alk-1-enyl ethers continue to increase while alkyl-ether radioactivity has declined to half its peak level.

The most suggestive evidence of alkyl ethers as direct precursors of plasmalogens comes from the ^{3}H-chimyl alcohol incubations. Here 25% of the added radioactivity appears in alk-1-enyl ethers by 9 hr. Comparing these results with those from the ^{14}C-palmitate study, it seems clear that this high labeling cannot be accounted for by the relatively small amount of alkyl ether cleavage yielding fatty acids.

Most of the radioactive alkyl and alk-1-enyl ethers are located in the ethanolamine phosphoglycerides and the unknown compound described in the accompanying paper (1). If, as we suspect, the unknown lipid is the ether analog of phosphatidic acid, this may be the intermediate involved in the desaturation step. Unfortunately, the instability of the unknown under these conditions has not allowed a detailed analysis to be made. However, considering the relatively small amount of alk-1-enyl ether found in this lipid as compared with the ethanolamine phosphoglycerides, the specific radioactivity of the unknown plasmalogen must be much higher than that of its ethanolamine analog.

The preliminary in vitro incubations attempted so far have been unsuccessful in effecting plasmalogen synthesis. The fact that ^{14}C-palmitate is rapidly incorporated suggests that further experimentation may produce a cell-free system capable of measurable plasmalogen biosynthesis.

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Effects of Su-13437, A New Hypolipidemic Drug, Upon Synthesis In Vivo of Hepatic and Carcass Total Fatty Acids and Total Cholesterol

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ABSTRACT

The present study is directed toward obtaining information on the metabolic effects and on the mode of action of CIBA-Su-13437, a new hypolipidemic phenolic ether structurally related to clofibrate. The ability of Su-13437 to affect the net formation of lipids in vivo was studied by measuring the total incorporation of intraperitoneally injected 1,2-^{14}C-sodium acetate and uniformly labeled ^{14}C-glucose into total fatty acids and total cholesterol by the liver and carcass of control and Su-13437-treated mice. Treated mice received Su-13437 orally at 25, 100 or 250 mg/kg/day for 14 or 15 consecutive days. Treatment with Su-13437 resulted in pronounced enlargement of the liver and significant reductions in the plasma levels of triglycerides and total cholesterol. Essentially all of the observed metabolic effects of Su-13437 were confined to the liver. At all doses studied, in addition to liver enlargement, there were large increases in incorporation of both ^{14}C-acetate and ^{14}C-glucose carbon into hepatic total fatty acids; marked increases in hepatic total fatty acid content; and decreases in the liver's relative cholesterol content of up to 30% with little or no effect upon the net synthesis of cholesterol per gram of liver. These observations are interpreted as indicating that Su-13437 lowers plasma triglyceride and cholesterol levels by means other than net inhibition of fatty acid and cholesterol formation by either hepatic or extrahepatic tissues.

INTRODUCTION

CIBA-Su-13437 [2-methyl-2-(*p*-1,2,3,4-tetrahydro-1-naphthyl)-phenoxy)propionate] is a recently introduced phenolic ether, structurally related to clofibrate (ethyl-*p*-chlorophenoxyisobutyrate), which is reported to possess pronounced hypolipidemic properties in experimental animals (1,2) and man (3,4). Su-13437 is active at lower doses than clofibrate and decreases both the circulating levels of cholesterol and triglycerides, although the effects on glyceride levels seem to predominate. In contrast to clofibrate, which has been extensively investigated for almost 10 years, there is little information available on the biochemical effects of Su-13437 and on the possible mechanisms by which it lowers plasma lipid levels. The present study is directed toward obtaining information on the mode of action of Su-13437. Specifically, the objective is to measure the effects of this drug upon the synthesis of total fatty acids and total cholesterol by the liver and carcass of the intact animal. The immediate interest in these metabolic processes as possible sites of action for Su-13437 stems from the recent observations of Maragoudakis (5,6) that Su-13437 and clofibrate inhibit the activity of purified hepatic acetyl CoA carboxylase (an early enzyme in fatty acid synthesis) and the earlier reports (7,8) that clofibrate inhibits hepatic cholesterol synthesis.

MATERIAL AND METHODS

Treatment of Mice

Male mice (30 g, Swiss Webster, from Hilltop Lab Animals, Inc., Scottdale, Pa.) maintained on a standard Purina rat and mouse lab chow diet (6.3% fat) received intragastrically Su-13437 suspended in 1% aqueous gelatin at 25, 100 or 250 mg/kg once daily for 14 or 15 consecutive days. The highest dose studied, 250 mg/kg, corresponds to a dosage level at which clofibrate has frequently been investigated in animals (7,8). Age- and weight-matched, untreated male mice also maintained on the mouse chow diet served as controls.

Two hours after receiving the last dose of the drug on day 15, treated mice or control mice received by intraperitoneal injection 1,2-^{14}C-sodium acetate at 0.1 μCi/g body weight (sp. act. = 140 μCi/mM) or uniformly labeled ^{14}C-glucose at 0.2 μCi/g body weight (sp. act. = 137 μCi/mM). The mice thus receive either about 1.8 mg of labeled acetate or about 8 mg of labeled glucose. Food and water were removed from both control and treated mice for the 2 hr immediately prior to

TABLE I

Effects of Su-13437 Upon Plasma Cholesterol and Plasma Triglyceride Levels[a,b].

Group	Total cholesterol				Triglycerides			
	Number of mice	Mg/100 ml	Per cent change	P	Number of mice	Mg/100 ml	Per cent change	P
Control	31	128 ± 18	—	—	18	66.2 ± 22.0	—	—
Su-13437								
25 mg/kg	18	115 ±26	-10	<0.07	18	36.9 ± 8.1	-44	<0.001
100 mg/kg	23	107 ± 20	-16	<0.001	15	39.9 ± 9.9	-40	<0.001

[a]Mice were treated with Su-13437 orally at 25 or 100 mg/kg once daily for 14 consecutive days.
[b]Values are means ± one standard deviation.

administration of the labeled substrates. After injection of the radioisotopes, the mice were then placed into metabolism chambers for a 3 hr incubation in vivo. Subsequently, the mice were killed by cervical dislocation and the liver and carcass (all extrahepatic tissues) were separated. The liver and carcass were separately saponified in ethanolic KOH and the total fatty acids and total cholesterol were then separately extracted into petroleum ether as indicated previously (9). The total cholesterol, isolated as the digitonide (10), and the isolated total fatty acids were assayed for ^{14}C content by liquid scintillation counting. The cholesterol digitonide precipitates were prepared for scintillation counting by the method of Avoy et al. (7). Total fatty acids and digitonide precipitates were measured gravimetrically. The measure of lipid synthesis is the per cent of the injected ^{14}C-acetate or ^{14}C-glucose radioactivity incorporated into the total fatty acids and total cholesterol of the liver and carcass.

Measurement of Plasma Lipids

Immediately after cervical dislocation of control or treated mice, blood was drawn by cardiac puncture into 1 ml syringes containing 0.19 ml of a citrate anticoagulant. Total blood volume was recorded and a microhematocrit was done on each sample. The plasma, separated by centrifugation, was extracted in isopropanol (0.25 ml of plasma + 4.75 ml of isopropanol). After removal of phospholipids and glucose from these extracts with Zeolite (11), aliquots of the extracts were directly assayed for total cholesterol content by an automated method (12). Glyceride glycerol was released by saponification and then measured essentially as described by Lofland (13). In all cases the proper corrections were made for the dilution of the plasma by the citrate anticoagulant.

RESULTS

Both 25 and 100 mg/kg of Su-13437 given orally to mice once daily for two weeks resulted in about 40% reductions in the plasma triglyceride levels (Table I). The effects on plasma total cholesterol concentrations were much less pronounced. Su-13437 given at 100 mg/kg for two weeks decreased the plasma cholesterol levels by only an average 16%, $P(t)<0.001$; 25 mg/kg produced a decrease (10%) which was only of borderline statistical significance (Table I).

The effects of Su-13437 upon liver weight and upon liver and carcass lipid content were also measured (Table II). At all drug doses studied, both the absolute liver weight and the weight relative to the whole mouse greatly increased, ranging from a 60% increase at 25 mg/kg to about a 200% increase at 250 mg/kg. By way of comparison, Hess and Bencze (1) observed a 100% increase in the relative liver weight of rats treated orally with 100 mg/kg of Su-13437 for 14 days.

With regard to lipid content, Su-13437 had no statistically significant effects upon the total fatty acid or cholesterol content of the carcass. However, drug treatment resulted in a pronounced increase in the total fatty acid content of the whole liver. The increase is explained on the basis of liver enlargement since the total fatty acid content per gram of liver did not change. Of particular significance, treatment with Su-13437 caused a significant decrease in the hepatic relative cholesterol content, i.e., milligram cholesterol per gram liver wet weight. At 100 and 250 mg/kg there was a decrease of about 30%. At 25 mg/kg the decrease, although statistically significant, amounted to only 12%. The total cholesterol content of the whole liver increased with drug treatment since the increase in liver size more than compensated for the decrease in the cholesterol content per gram of

TABLE II

Effects of Su-13437 Upon Liver Weight and Upon Liver and Carcass Lipid Content[a]

Dose of drug, mg/kg[b]	Liver			Total fatty acid content, mg			Total cholesterol content, mg		
	Number of mice	Total weight g	Per cent body weight	Carcass[d]	Whole liver	Per g liver	Carcass	Whole liver	Per g liver
0[c]	11	1.77± 0.34	5.69± 0.85	1838± 553	61.4± 16.6	35.3± 11.5	69.7± 5.3	4.55± 0.68	2.50± 0.40
25	13	2.85±[e] 0.74	9.21±[e] 1.48	1940± 1028	105.6±[e] 27.8	37.4± 5.1	69.0± 8.8	6.08±[e] 1.03	2.19±[g] 0.31
100	4	4.01±[e] 0.62	12.64±[e] 1.94	1605± 659	160.1±[e] 45.9	39.4± 6.1	62.8± 4.9	7.13±[f] 1.75	1.76±[e] 0.19
250	9	5.80±[e] 0.67	16.33±[e] 0.85	1916± 855	239.2±[e] 36.0	41.4± 4.6	62.8± 7.6	9.98±[e] 1.70	1.72±[e] 0.20

[a]Values are means ± one standard deviation.
[b]Su-13437 was given orally at 25, 100 or 250 mg/kg/day for 15 days.
[c]Untreated control mice.
[d]Refers to the whole mouse minus the liver.
[e]P(t) of difference from control <0.005.
[f]P(t) <0.02.
[g]P(t) <0.05.

liver. These results partially agree with those published by Hess and Bencze (1) for the rat. They report that 100 mg/kg of Su-13437 given orally for 14 days caused a 25% to 40% decrease in the free and esterified cholesterol content per gram of liver while the free cholesterol content of the whole liver increased by about 80%.

Table III presents data on the effects of 25, 100 and 250 mg/kg of Su-13437 upon the formation of total fatty acids and total cholesterol from ^{14}C-acetate. The data is expressed as the per cent of the injected ^{14}C-acetate incorporated into the total fatty acids and total choelsterol of the carcass and liver. Drug treatment had little or no effect upon the incorporation of ^{14}C-acetate into carcass fatty acids and cholesterol. In all cases about 3% of the injected ^{14}C-acetate was incorporated into the total fatty acids of the carcass. In contrast, there was a very large increase in incorporation of ^{14}C-acetate into the total fatty acids of both the whole liver and per gram of liver at all drug doses studied. For example, the whole liver of untreated mice incorporated about 0.4% of the injected ^{14}C-acetate into hepatic total fatty acids. This represented about 12% of the total fatty acid synthesis observed for the whole mouse. However, when mice received, for example, 100 mg/kg of Su-13437 the total liver incorporated almost nine times as much of the labeled substrate into total fatty acids, about 3.4% of the injected ^{14}C-acetate and this now represented more than 50% of the total fatty acid synthesis measured for the entire mouse. The magnitude of the increased incorporation into fatty acids per gram of liver was seen to be independent of drug dose. In all cases it increased by more than a factor of four. In comparison to fatty acid synthesis, the effects of Su-13437 upon the formation of total cholesterol by the liver were less pronounced. The drug caused no significant change in cholesterol formation from ^{14}C-acetate per gram of liver except at the highest dose. Here there was a slight increase. Incorporation into cholesterol of the whole liver was significantly increased at 100 and 250 mg/kg. There was also little effect upon the formation of carcass cholesterol. The indication of a small but significantly depressed incorporation into carcass cholesterol at 25 mg/kg was not supported by subsequent experiments.

Although ^{14}C-acetate is a convenient tool for investigating fatty acid and cholesterol synthesis it is not a natural exogenous substrate for lipid formation in man or mouse. Glucose is undoubtedly the main carbon source for lipogenesis under usual conditions (14). For this reason experiments were conducted with ^{14}C-glucose to see whether they could confirm the observations on lipid synthesis from ^{14}C-acetate. As was seen with ^{14}C-acetate, treatment with Su-13437 increased the net formation of total fatty acids by the whole liver and per gram of liver from uniformly labeled ^{14}C-glucose (Table IV). As before, the drug had no effect upon fatty acid formation by the

TABLE III

Effects of Su-13437 Upon Fatty Acid and Cholesterol Synthesis in vivo from 1,2-^{14}C-Acetate, Given i p at 0.1 μCi/g Body Weight.

Dose of drug, mg/kg[b]	Number of mice	Per cent of injected ^{14}C-acetate incorporated into total fatty acids and total cholesterol at 3 hr after injection[a]					
		Total fatty acids, %			Total cholesterol, %		
		Carcass	Whole liver	Per g liver	Carcass	Whole liver	Per g liver
0	6	2.81± 0.41	0.383± 0.130	0.196± 0.062	0.519± 0.082	0.050± 0.018	0.025± 0.008
25	7	3.22± 0.65	2.200±[c] 0.537	0.916±[c] 0.242	0.400±[e] 0.103	0.064± 0.037	0.026± 0.015
100	4	3.01± 0.78	3.365±[c] 1.106	0.861±[c] 0.345	0.540± 0.098	0.138±[d] 0.056	0.035± 0.014
250	4	3.38± 0.98	5.417±[c] 1.907	0.901±[d] 0.340	0.637± 0.023	0.253±[c] 0.061	0.042±[e] 0.011

[a]Values are means ± one standard deviation.
[b]Su-13437 was given at 25, 100, or 250 mg/kg/day for 15 days. 0 drug dose refers to untreated controls.
[c]$P(t) < 0.005$.
[d]$P(t) < 0.02$.
[e]$P(t) < 0.05$.

carcass at 25 mg/kg, although 250 mg/kg seemed to produce a slight but statistically significant inhibition. With regard to cholesterol formation, 25 mg/kg did not effect cholesterol synthesis from ^{14}C-glucose by the liver or carcass. As observed with ^{14}C-acetate, 250 mg/kg stimulated cholesterol formation by the liver but not by the carcass. Thus with slight exception, the overall effects of Su-13437 upon lipid synthesis from ^{14}C-acetate and ^{14}C-glucose are the same. With both isotopes at low and high drug doses there was stimulation of fatty acid formation by both the whole liver and per gram of liver; cholesterol formation by the liver from both isotopes was unaffected at 25 mg/kg but stimulated at 250 mg/kg; and lastly drug treatment resulted in only small if any effects upon the synthesis of total fatty acids and cholesterol by the carcass. In part, these findings are in close agreement with the observations presented by Duncan and Best (15) in a 1969 abstract. They reported that Su-13437 and to a lesser extent clofibrate significantly increased incorporation of orally administered 1-^{14}C-sodium acetate into hepatic total fatty acids of the rat. As observed in the present study, the stimulation of hepatic fatty acid synthesis may also go along with the finding of Hess et al. (2) that Su-13437, orally given to rats at 100 mg/kg for 14 days, stimulated the incorporation of intravenously injected 1-^{14}C-palmitate into liver triglycerides.

Since there was some concern that the patterns of incorporation of radioactivity into body lipids as observed at 3 hr could have appreciably changed with greater time after substrate administration, experiments were conducted measuring the incorporation of ^{14}C-acetate into hepatic and carcass lipids at 9 hr after isotope administration to control and 25 mg/kg treated mice (Table V). As seen at 3 hr (Table III), there is an increase in the incorporation of ^{14}C into total fatty acids of both the whole liver and per gram of liver with no effects upon the extrahepatic fatty acid synthesis. Also in agreement, 25 mg/kg of Su-13437 still produced no changes in the formation of hepatic cholesterol from ^{14}C-acetate. Although there was less ^{14}C-acetate incorporated into carcass cholesterol by the treated mice at 9 hr, the difference was not statistically significant as it was at 3 hr. These results indicate that the experiments conducted at 3 hr are essentially representative of the patterns of incorporation for many hours after isotope administration. Throughout the time intervals studied, the principal observed drug effect was stimulation of hepatic fatty acid formation from both ^{14}C-acetate and ^{14}C-glucose.

Theoretically, one of several mechanisms could have accounted for the drug related increases in incorporation of 1,2-^{14}C-acetate and U-^{14}-glucose carbon into hepatic total fatty acids. These possibilities include, for

TABLE IV

Effects of Su-13437 Upon Fatty Acid and Cholesterol Synthesis in Vivo From U-^{14}C-Glucose, Given i pat 0.2 μCi/g Body Weight.

		Per cent of injected U-^{14}C-glucose incorporated into total fatty acids and total cholesterol 3 hrs after injection[a]					
		Total fatty acids, %			Total cholesterol, %		
Dose of Drug mg/kg[b]	Number of mice	Carcass	Whole liver	Per g liver	Carcass	Whole liver	Per g liver
0	5	1.49± 0.16	0.016± 0.015	0.0088± 0.0055	0.111± 0.022	0.0018± 0.0013	0.0010± 0.0006
25	6	1.32± 0.31	0.072±[c] 0.045	0.0202±[d] 0.0085	0.128± 0.045	0.0028± 0.0018	0.0008± 0.0006
250	5	1.15±[d] 0.20	0.562±[e] 0.264	0.0996±[e] 0.0454	0.100± 0.010	0.0755±[c] 0.0324	0.0135±[c] 0.0050

[a]Values are means ± one standard deviation.
[b]Oral dose per day for 15 days. 0 dose refers to control mice.
[c]$P(t) < 0.025$.
[d]$P(t) < 0.05$.
[e]$P(t) < 0.005$.

example, decreased pool sizes of hepatic acetate and glucose in treated mice, inhibition of fatty acid catabolism or an overall stimulation of the liver's fatty acid synthesizing enzymes. This last possibility may deserve special consideration since both Su-13437 and clofibrate cause a cytoplasmic hypertrophy of the liver (2) and this hypertrophy, at least for clofibrate, includes a marked increase in the liver's cell sap protein concentration (16).

DISCUSSION

The use of the mouse as a model for studies of hypolipidemic drugs requires certain special considerations since the lipid metabolism of the mouse is apparently highly dependent upon its genetic background. Inbred strains vary greatly in their susceptibility to develop obesity, ranging from extremely resistant to highly susceptible and also in the levels of their plasma cholesterol (17). In general, strains highly susceptible to obesity possess elevated plasma cholesterol levels. Bruell et al. (18) observed that in five inbred strains of mice the plasma cholesterol ranged from 128 mg/100me to 208 mg/100 me. Considering this information, the mice used in the present study are probably of the resistant to moderately resistant genetic type since they possessed relatively low plasma total cholesterol levels (128 ± 18 mg/100 ml). The effectiveness of Su-13437 as a hypo-

TABLE V

Influence of Time Upon the Effects of Su-13437 on Fatty Acid and Cholesterol Synthesis in Vivo From 1,2-^{14}C-Acetate

		Per cent of injected 1,2-^{14}C-acetate incorporated into total fatty acids and total cholesterol at 9 hr after injection[a]					
		Total fatty acids, %			Total cholesterol, %		
Dose of drug, mg/kg[b]	Number of mice	Carcass	Whole liver	Per g liver	Carcass	Whole liver	Per g liver
0	3	2.76± 1.06	0.258± 0.195	0.164± 0.144	0.523± 0.073	0.031± 0.020	0.017 ± 0.009
25	3	2.84± 0.21	1.466±[c] 0.451	0.605±[d] 0.183	0.476± 0.070	0.041 0.015	0.018± 0.010

[a]Values are means ± one standard deviation.
[b]Oral dose per day for 15 days. 0 dose refers to control mice.
[c]$P(t) < 0.02$.
[d]$P(t) < 0.05$.

lipidemic drug for the strain of mice used in these studies was established by significant reductions in serum cholesterol and triglyceride levels following two weeks oral dosage with 25 and 100 mg/kg/day of this drug. Confirming other reports (2-4) reductions of plasma triglyceride levels were seen to exceed those of plasma cholesterol.

Essentially all of the observed metabolic effects of Su-13437 in the mouse were confined to the liver. These involved enlargement of the liver, stimulation of hepatic fatty acid formation from both ^{14}C-acetate and ^{14}C-glucose and reductions of the liver's relative cholesterol content. There was generally little effect upon the synthesis of total cholesterol per gram of liver. These observations permit some general comments on the possible mode of hypocholesterolemic and hypotriglyceridemic action of Su-13437. First, Su-13437 in contrast to clofibrate would not appear to lower plasma cholesterol levels secondary to an overall inhibition of either hepatic or extrahepatic cholesterol synthesis. Other possibilities, such as acceleration of cholesterol catabolism and excretion or tissue redistribution, or both, could account for the observed reductions in hepatic and plasma cholesterol levels.

With regard to triglycerides, the depression of plasma triglycerides by Su-13437 in the mouse certainly does not seem to involve a net inhibition of hepatic or extrahepatic fatty acid de novo synthesis. The lack of inhibition of total extrahepatic fatty acid synthesis may be the more significant observation since most of the total fatty acid synthesis by the whole animal occurs outside of the liver (14). This idea is confirmed by the present study where in control mice about 88% of the animal's total fatty acid synthesis from ^{14}C-acetate and over 98% of the synthesis from ^{14}C-glucose occurred outside the liver. The observed absence of inhibition in vivo of fatty acid synthesis by Su-13437 reported here and earlier by Duncan and Best (15) is not consistent with the suggestion by Maragoudakis (6) that Su-13437 lowers total body lipids by inhibiting overall fatty acid formation secondary to inhibition of acetyl-CoA carboxylase. Likewise, Maragoudakis' suggestion that clofibrate also exerts its hypolipidemic action through inhibition of fatty acid synthesis seems to disagree with the report of Gould et al. (19) showing that clofibrate given to rats greatly stimulates hepatic triglyceride synthesis from injected ^{14}C-acetate and increases hepatic triglyceride content. Lastly, Boberg et al. (4) recently reported measurements of triglyceride turnover in the Su-13437-treated human which were interpreted to indicate that this drug does not significantly decrease triglyceride production. Boberg and coworkers (4) suggest that Su-13437 lowers plasma triglyceride levels by increasing plasma clearance of triglycerides by the peripheral tissues. In conclusion, the likelihood that Su-13437 exerts its hypolipidemic actions by means other than net inhibition of fatty acid and cholesterol synthesis is indicated by the findings of the present study.

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The Lipids of Krill (*Euphausia* Species) and Red Crab (*Pleuroncodes Planipes*)

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ABSTRACT

The composition of the lipids of two samples of krill and one of "red crab" was determined by thin layer, column and gas chromatographic procedures. A large number of unusual fatty acids were present.

INTRODUCTION

In a previous communication we reported the proximate analyses of two samples of krill (*Euphausia* species) and one of "red crab", a galatheid decapod (*Pleuroncodes planipes*) (1). These animals are important links in the food chain of whales and tuna fish and may have promise as new food resources for man. Details of the nature of their lipids are reported in this paper. Column and thin layer chromatography (TLC) were used for fractionation and identification of the individual components. Fatty acids were determined, after hydrolysis and methylation, by gas chromatography. The fats contained a large number of unusual fatty acids.

EXPERIMENTAL PROCEDURES

The samples of krill (*E. pacifica* (from Eureka, California) and *E. superba*) and of red crab (*P. planipes*) were obtained and the lipids extracted as previously described (1). After purification by Sephadex chromatography (2), the components were fractionated by silicic acid column chromatography as follows: silicic acid (Mallinckrodt, AR-CC7 200-325 mesh, activated 24 hr at 100 C) was packed into a 1 in. all glass Teflon column (Model LC-1 Chromatronix, Berkeley, California) equipped with 10 μ Teflon cloth bed supports, slurried in 2:1 (v/v) chloroform-methanol. Samples were introduced with a sample injector (Chromatronix Model SU-8031) and pumped (Chromatronix Model CMP-1) at a constant flow rate (120 ml/min). The chromatography system is constructed so that the solvent comes into contact only with glass and Teflon, thus reducing the risk of contamination by metals and plasticizers. Oxidation is minimized by deoxygenating the solvents prior to pumping and collecting all samples under N_2. The columns were eluted by a stepwise gradient of chloroform-methanol (3). Fractions were collected in bulk (300-400 ml), and reduced under vacuum on a flash evaporator. The lipids were redissolved in chloroform and stored in solution under N_2 at -10 C prior to analysis.

Fractions were identified by IR spectra and TLC (4). For TLC runs samples were spotted in a stream of N_2 in an application box (Model DB Brinkman Instruments, Westbury, N.Y.), and compared with standards (Applied Science, State College, Pa.) after charring.

Fatty acid composition of the major fractions was determined as follows. Duplicate aliquots were saponified and methylated as described previously (5). The resulting esters were analyzed on a Hewlett Packard Model 810 gas liquid chromatograph. A 6 ft column, 4 mm i.d., packed with 10% diethylene glycol adipate on Chromsorb Q, 80 to 100 mesh (Applied Science) was employed at 190 C with a N_2 flow rate of 75 ml/min. The column was standardized with purified methyl esters (Applied Science) as described by Horning et al. (6), and the amount represented by each peak was estimated by the method of Carroll (7). Samples were hydrogenated (8) and rerun to help identify the fatty acids.

Solvents were fractionally distilled over a 15 plate Oldershaw column and deoxygenated prior to usage. All extractions were carried out in an inert atmosphere of either CO_2 or N_2, and samples were stored under N_2.

RESULTS

Whole *E. pacifica*, *E. superba* and *P. planipes* contained 23.8%, 18.7% and 7.6% total lipid respectively on a dry basis (1). The amount of lipids in the red crab meat plus viscera (shell-free) was about 30% as estimated indirectly from the chitin values (1).

Silicic acid chromatography data are shown in Table I. *E. superba* had roughly 10% more phospholipid than *E. pacifica* and was also richer in phosphatidyl choline (PC). All samples contained 3-5% lysophospholipids which may

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TABLE I

Lipid Composition

Fraction No.	Constituents[a]	Yield, per cent of applied sample		
		Red crab	*Euphausia* sp.	
		P. planipes	*E. superba*	*E. pacifica*
1	NL[b], FFA, sterols	55.3	59.8	66.5
2	PE	6.6	6.1	4.5
3	PS, Lyso PE	6.7	1.6	1.4
4	PC, some Sph	26.4	30.9	21.0
5	Sph, Lyso PC	1.9	1.6	3.0
Total recovered		96.9	100.0	96.4

[a]By TLC, see text.
[b]Abbreviations: NL, neutral lipids; FFA, free fatty acids; PE, phosphatidyl ethanolamine; PS, phosphatidyl serine; Lyso PE, lysophosphatidyl ethanolamine; PC, phosphatidyl choline; Sph, sphingolipids; Lyso PC, lysophosphatidyl choline.

TABLE II

Fatty Acid Composition of *E. superba* Fractions[a]

Fatty acid carbon chain, double bonds	Fraction 1 (NL),[b] %	Fraction 4 (PC), %	Fraction 2 (PE), %
14:0	6.3	1.6	0.2
16:0	27.5	26.8	11.8
17:0	1.1	0.3	0.2
16:1 + anteiso 17:0	12.0	4.3	1.4
18:0	2.0	1.8	1.4
18:1 + anteiso 19:0	16.7	12.2	10.1
anteiso 21:0 + 20:1	1.5	0.6	0.1
20:3	0.8	1.2	1.8
20:5	19.9	33.6	25.0
22:6	9.3	13.6	41.1

[a]See Table I and text for identification of fractions. Additional peaks which individually accounted for 1% or less of the total fatty acids were tentatively identified as: iso 14:0, 15:0, anteiso 15:0, iso 16:0, iso 18:0, 18:2 and/or 19:0, 18:3, 18:4 and/or 20:0, iso 20:0, 20:2, iso 20:5, iso 20:3 and/or 22:0, 22:2 and/or 23:0, 22:5.
[b]Abbreviations: see Table I.

TABLE III

Fatty Acid Composition of *E. Pacifica* Fractions[a]

Fatty acid carbon chain, double bonds	Fraction 1 (NL),[b] %	Fraction 4 (PC), %	Fraction 2 (PE), %
14:0	4.9	1.3	0.3
16:0	22.5	25.7	15.1
iso 16:0	0.7	0.1	2.4
16:1 + anteiso 17:0	10.2	3.8	1.6
18:0	1.9	1.8	2.2
18:1 + anteiso 19:0	15.3	12.0	10.4
18:2 + 19:0	1.0	0.9	1.3
20:0 + 18:4	1.3	0.7	1.1
20:3	1.2	1.2	2.9
20:5	22.8	32.5	22.0
22:6	10.9	15.5	34.6

[a]See Table I and text for identification of fractions. Additional peaks which individually accounted for 1% or less of the total fatty acids were tentatively identified as: iso 14:0, 15:0, anteiso 15:0, 17:0, iso 18:0, 18:3, iso 20:0, 20:1 and/or anteiso 21:0, 20:2, iso 20:5, 21:0 and/or iso 20:2, 22:0 and/or iso 20:3, 23:0 and/or 22:2, 22:3, 22:5.
[b]Abbreviations: See Table I.

TABLE IV

Fatty Acid Composition of Red Crab Fractions[a]

Fatty acid carbon chain, double bonds	Fraction 1 (NL),[b] %	Fraction 4 (PC), %	Fraction 2 (PE), %
14:0	5.0	1.4	0.3
15:0	1.2	0.3	0.2
16:0	16.6	25.3	7.2
iso 16:0	1.0	0.2	5.7
16:1 + anteiso 17:0	5.7	3.8	2.1
17:0	1.3	0.1	1.6
18:0	2.8	1.8	5.7
iso 18:0	0.9	0.2	5.7
18:1 + anteiso 19:1	14.1	11.7	6.4
19:0 + 18:2	2.0	1.1	1.9
18:4 + 20:0	1.8	0.8	0.2
20:1 + anteiso 21:0	1.1	0.7	0.5
20:3	2.6	1.3	4.5
20:5	12.1	33.5	25.5
iso 20:3 + iso 22:0	0.9	0.2	1.4
22:3	---	0.4	1.2
24:0 + 22:4	3.1	0.7	tr
22:6	22.4	14.8	26.7

[a]See Table I. Additional peaks which accounted individually for 1% or less of the total fatty acids were tentatively identified as: iso 14:0, anteiso 15:0, 18:3, iso 20:0, 20:2, iso 20:5, iso 20:2 and/or 21:0, 22:2 and/or 23:0, 22:5.

[b]Abbreviations: See Table I.

be in part due to delays in handling. TLC showed about 1-2% phosphatidyl inositide (PI) and trace amounts of phosphatidyl serine (PS) and sphingolipids (Sph). Thus the phospholipid fraction of the krill samples consisted mainly of PC (about 75%) with some phosphatidyl ethanolamine (PE).

The phospholipids of whole red crab were more complicated. The ratio of PC to PE was the same as that found in the krill, but the crab contained PS in an amount equal to PE. There were also moderate amounts of diphosphatidylglycerol (DPG), a small amount of Sph, and trace amounts of lysophospholipids. No PI was detected.

By TLC krill Fraction 1 (Table I) consisted mainly of free fatty acids and triglycerides with lesser amounts of sterol and sterol esters; mono- and diglycerides were absent. Red crab Fraction 1 had large amounts of free fatty acid and moderate amounts of sterols as the major components. A small amount of triglycerides but no mono- or diglycerides were present. Quantitative data on these fractions were not obtained.

The fatty acid composition of the major components are summarized in Tables II-IV. Table V presents the results of hydrogenation of the fatty acids of red crab PE. There were more components in the hydrogenated methyl esters than were apparent in the same esters before hydrogenation (Table IV).

The krill components differed considerably in fatty acid composition (Tables II and III). Fractions 1 and 4 had about twice the amount of 20:5 over 22:6, but fraction 2 had 1 1/2 times as much 22:6 as 20:5. Fractions 1 and 4 both contained about 25% 16:0; the PE Fraction 2, about 12-15%. On the other hand, Fraction 1 had more 16:1 than did the two others.

Crab Fractions 1 and 4 were similar to those

TABLE V

Fatty Acid Composition of Red Crab Hydrogenated Phosphatidyl Ethanolamine

Fatty acid carbon chain, double bonds	Hydrogenated fraction 2,[a] %	Calculated from Table IV, %
16:0	7.5	9.3
iso 16:0	5.5	5.7
17:0	1.8	1.6
anteiso 17:0	1.7[b]	---
18:0	14.7	15.3
iso 18:0	8.9	5.7
20:0	29.2	30.7
iso 20:0	1.2	0.5
22:0	26.3	28.4

[a]See Table IV for identification. Additional peaks which individually accounted for 1.0% or less of the total fatty acids were: 14:0, iso 14:0, 15:0, anteiso 15:0, 19:0, anteiso 19:0, 21:0, anteiso 21:0, iso 22:0, 23:0, 24:0.

[b]This fatty acid or its unsaturated precursor would have been masked by other peaks in runs with unhydrogenated fractions as would also 19:0, anteiso 19:0, anteiso 21:0, iso 22:0, 23:0, 24:0.

found in krill except that the 20:5/22:6 ratio for neutral lipids (NL) was reversed (i.e., twice as much 22:6 as 20:5). Fraction 2 was entirely different. Along with the normal straight chain fatty acids there was a branch chain series which was abundant in C16 and C18 fatty acids. Identification of the unsaturated acids of this series was rendered difficult by a lack of standards and by the fact that they were masked by the other fatty acids. Hydrogenation was useful in sorting out their relative abundance (Table V). The data indicated that the iso 16 is apparently saturated while a third of the iso 18 is probably unsaturated. The unsaturated iso 18 acid may be masked by 18:0 under normal gas liquid chromatography (GLC) conditions. Also the 16:1 peak may have been masking anteiso 17:0 which was not detected in the normal chromatogram. Hence, the assignments shown in Tables II-V are tentative for the minor components listed in the footnotes. The PE fractions of the krill also contained small amounts of these branched chained isomers. The number of unsaturated fatty acids together with odd numbered and branched chain fatty acids present make these lipids difficult to analyze by conventional GLC.

DISCUSSION

Euphausids

The amount of lysophospholipids present and the high amount of free fatty acids (20-30% by TLC) indicates that hydrolysis had occurred during storage and handling. However there were no detectable mono- or diglycerides and the relatively low amounts of lysophospholipids could not alone account for the high fatty acid content. Maksimov (9) also found a high fatty acid content by titration of the lipids of freshly caught krill. However Ackman and Eaton (10) found little free fatty acids in freshly caught shrimp. Hydrolysis could occur by bacterial or intestinal enzymes or both. Since there is little evidence as to which of the natural components might be selectively attacked, the compositions as recorded may well reflect unusual sets of circumstances. Further studies on freshly caught samples are needed to elucidate the composition as captured. Seasonal variations in crustacea lipid also need further study.

The unusually high contents of 20:1 and 22:1 found by Ackman and Eaton (9) in the North Atlantic euphausid *Meganyctiphanes norvegica* were not observed in the euphausids studied herein. The amount of unsaturated lipids observed by Nonaka and Koizumi (10) was low (3-6%) in 22:6. However these authors obtained krill from whale stomachs and hydrolysis and oxidation of their lipids could have occurred.

Red Crab

Fraction 1 was similar in composition to those reported for krill except that it contained about 10% sterols. The approximately 30% free fatty acid content again indicates hydrolysis.

The phospholipid pattern showed that the amounts of PE and PC were similar but there was relatively a high content of PS (16% of the phospholipids). PS concentration seems to be species dependent (12) and seasonal variations might occur depending on such variables as eating habits.

The fatty acids included branched chain and odd number fatty acids and these were especially rich in the PE fraction. There appears to be a homologous series of branched chained fatty acids including the unsaturated 18 series. The nature of this series of isomers in particular tissue needs to be more thoroughly investigated. Since the PE fraction of the euphausids also contained some branch chain isomers, there is the possibility of a general occurrence of these isomers in many crustacea. The gas chromatographic procedure used in this study did not differentiate the unsaturated branched chain fatty acids from straight chain fatty acids; hence the branched chain unsaturated fatty acids were inferred from hydrogenation data (Table V). Probably the open tubular gas chromatographic technique would help to differentiate the various compounds.

Since this work was completed, two additional studies of the lipids of *E. superba* have appeared (13,14). The compositions of the major fatty acids are not too different from those reported here except that Sidhu et al. (14) found 13.2% of 22:5 and no 20:5. We report 20% 20:5 in Fraction 1 and more in the phospholipids and amounts of 22:5 less than 1%. Hansen and Meiklen (13) similarly find 16% 20:5 and 0.4% 22:5. An analytical or typographical error may be involved in the Sidhu et al. data.

In a recent paper containing compositional data on two species of North Atlantic krill, Ackman et al. (15) have provided an excellent summary of past investigations in this field.

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Cellularity of Adipose Tissue in Cold-Exposed Rats and the Calorigenic Effect of Norepinephrine

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ABSTRACT

Adipose tissue in rats maintained at normal ambient temperature grows by a mixture of hyperplasia and hypertrophy in the early growth stage of the rat. In the mature rat, the tissue grows primarily by hypertrophy. When the rat is acclimated to 5 C, growth of adipose tissue occurs primarily by hyperplasia throughout the body weight ranged studied. As a consequence adipose tissue from rats maintained in the cold has a much larger number of fat cells which are much smaller in size. In order to achieve the adaptation which permits hyperplasia to continue throughout the adult stage the animal must be exposed to cold at an early age, and it requires that the animal remain in the cold to continue. These changes in adipose tissue fat cell size and number are discussed in relation to the increased calorigenic and lipolytic effect of norepinephrine in cold-acclimated rats.

INTRODUCTION

At the International Symposium on Temperature Acclimation held in Buenos Aires in 1959, Masaro (1) suggested that the greatly increased level of plasma free fatty acids (FFA), induced by norepinephrine administration, might explain the marked calorigenic action of this neurohumor in the cold-acclimated rat. Since then, there has appeared considerable evidence to indicate that the increased calorigenic effect of norepinephrine in cold-acclimated rats might be due to their increased sensitivity to the lipolytic effect of norepinephrine (2-5). Previous work reported from this laboratory (6) indicated that in rats exposed to cold there is a definite increase in fat cell proliferation, resulting in adipose tissue containing a much larger number of cells. The amount of lipid deposited per cell, however, was significantly less in cold acclimated rats, resulting in adipose tissue which contained more fat cells, but much smaller in mass, than that from the control animals. It was also noted that the smaller fat cells from cold-acclimated rats are more sensitive to the lipolytic effect of norepinephrine then are the larger fat cells from rats maintained at normal ambient temperature.

If the calorigenic effect of norepinephrine is dependent on an increased mobilization of FFA from adipose tissue, which makes more FFA available for the skeletal muscle to oxidize, animals with smaller fat cells should respond to norepinephrine with a larger calorigenic effect. In the hope of shedding more light on the thermogenic response to cold, we measured the calorigenic effect of norepinephrine in growing rats maintained either at normal ambient temperature or in the cold. We also extended our previous observations (6), to consider under what conditions fat cell size and number can be affected by cold exposure.

MATERIALS AND METHODS

Male Sprague-Dawley rats weighing 50-75 g were individually caged in one of two environmental chambers and maintained at either 25 ± 2 C or 5 ± 1 C. All rats were fed Purina Lab Chow and water ad lib. The study consisted of three parts in which oxygen consumption of the intact rat and epididymal fat cell number and size were measured. In the first part, control rats housed at 25 C were killed by decapitation at intervals from one week to 14 weeks after being placed in the environmental chamber. Rats maintained at 5 C were kept in the cold chamber at least 6 weeks, and then killed at intervals over the next 10 weeks. In the second part, rats that were maintained at 5 C were removed when they reached a weight of 300 g, and transferred to the 25 C chamber. They were maintained at 25 C until they reached a weight of 450 g, at which time they were killed. In the third part, rats were maintained at 25 C until they reached a weight of 300 g. They were then transferred to the 5 C chamber and kept in the cold until they reached a weight of 475 g. Immediately after the rats were killed, both epididymal fat pads were excised, blotted and weighed. One pad was used for tissue lipid analysis while the contralateral pad served for the preparation of isolated fat cells, as previously described (6).

Oxygen consumption of the intact rat was

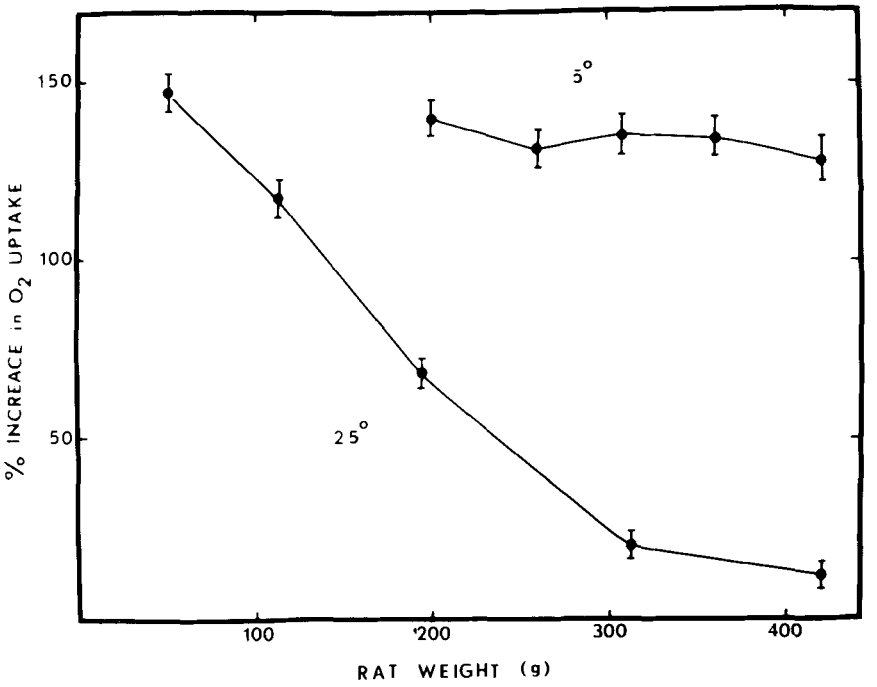

FIG. 1. The effect of age and cold acclimation on the calorigenic effect of norepinephrine. The per cent increase in oxygen uptake caused by the intraperitoneal injection of norepinephrine (0.2 mg/kg body weight) is plotted against the weight of the rats. Each point is the mean ± S.E.M. of six rats.

measured at 1 min intervals for a period of 1 hr in an open circuit system, consisting of a glass cylindrical chamber, connected to a Beckman E-2 oxygen analyzer, a wet test gas meter and appropriate controls for air flow rate. All measurements were conducted at an ambient temperature of 25 C, and values of oxygen consumption were corrected to standard temperature and pressure. After this control period of 1 hr the animal was injected intraperitoneally with norepinephrine (0.2 mg/kg body weight); oxygen consumption measurements were then obtained for an additional hour. The difference between the areas under the curves with and without norepinephrine was used to calculate the percent increase in oxygen consumption.

RESULTS

The calorigenic effect of norepinephrine in intact rats maintained at 5 and 25 C is depicted in Figure 1. The magnitude of the calorigenic effect depends on the age of the rat as well as the temperature at which the rat has been living. Whereas norepinephrine can tremendously increase the metabolic rate of young rats, its effect decreases progressively until by the time the rat weights 400 g little calorigenic response is obtained. Rats that have been cold-acclimated develop an accentuated calorigenic response to norepinephrine. The magnitude of this accentuated response in a cold-acclimated rat does not change appreciably with age. Thus the response of all cold-acclimated rats is as great as younger rats that have not been kept in the cold. If on the other hand rats are allowed to reach maturity before being exposed to cold, they do not develop this increased sensitivity to the calorigenic effect of norepinephrine (Table I).

Enlargement of adipose tissue mass may result from either cellular proliferation (hyper-

TABLE I

Calorigenic Effect of Norepinephrine in Normal and Cold-Exposed Rats

Rats	Per cent increase in O_2 uptake
Normal controls	15 ± 2[c]
Old cold-exposed[a]	21 ± 4
Young cold-exposed[b]	121 ± 7

[a]Rats maintained at 25 C till 300 g then placed at 5 C till 400 g.
[b]Rats maintained at 5 C from 100 g to 400 g.
[c]Mean ± S.E., six rats in each group.

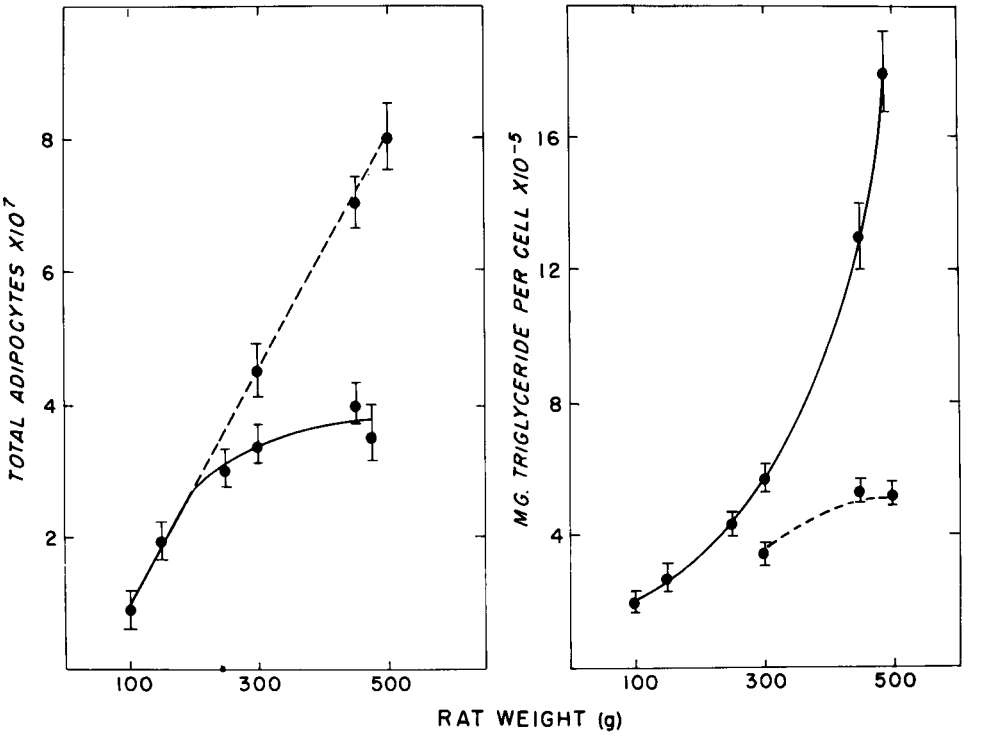

FIG. 2. The effect of age and cold acclimation on fat cell size and number in the epididymal fat pads of rats. The points on the solid line (——) are from animals kept at an ambient temperature of 25 C. Points on the broken line (- - - -) are from cold-acclimated rats maintained at 5 C.

plasia) or the deposition of increasing amounts of lipid within preexisting cells (hypertrophy). Figure 2 illustrates the results obtained from measurements of fat cell number and size as a function of the rat's growth at two environmental temperatures. These results are in excellent agreement with those from an earlier report (6). Whereas growth of adipose tissue in rats kept at normal ambient temperature is regulated almost solely by hypertrophy, rats kept in the cold from a very early age regulate adipose tissue growth primarily by hyperplasia. As a consequence adipose tissue from rats maintained in the cold has a much larger number of cells which are much smaller in mass.

If on the other hand rats are maintained at normal ambient temperature during their early growth stage, up to a body weight of 300 g, and then placed in a cold environment, the epididymal fat pads grow at a rate equivalent to that of rats kept in the cold from a very early age (Table II). However, growth in this case is due

TABLE II

Increase in Epididymal Fat Pad Mass and Its Lipid Content in Rats Whose Weight Increased From 300 to 475 g

Rats	No.	Body weight, g	Pad weight increase, g	Lipid weight increase, g
Exposed to 25 C	12	477 ± 5[a]	4.89 ± .48	4.51 ± .44
Adult cold-exposed[b]	16	472 ± 6	2.71 ± .28	2.42 ± .44
Young cold-exposed[c]	10	475 ± 8	2.83 ± .41	2.62 ± .41

[a]Mean ± S.E.

[b]Rats were maintained at 25 C until they reached a weight of 300 g. They were then placed in a 5 C chamber.

[c]Rats were placed in a 5 C chamber at a weight of 100 g and remained there throughout the experiment.

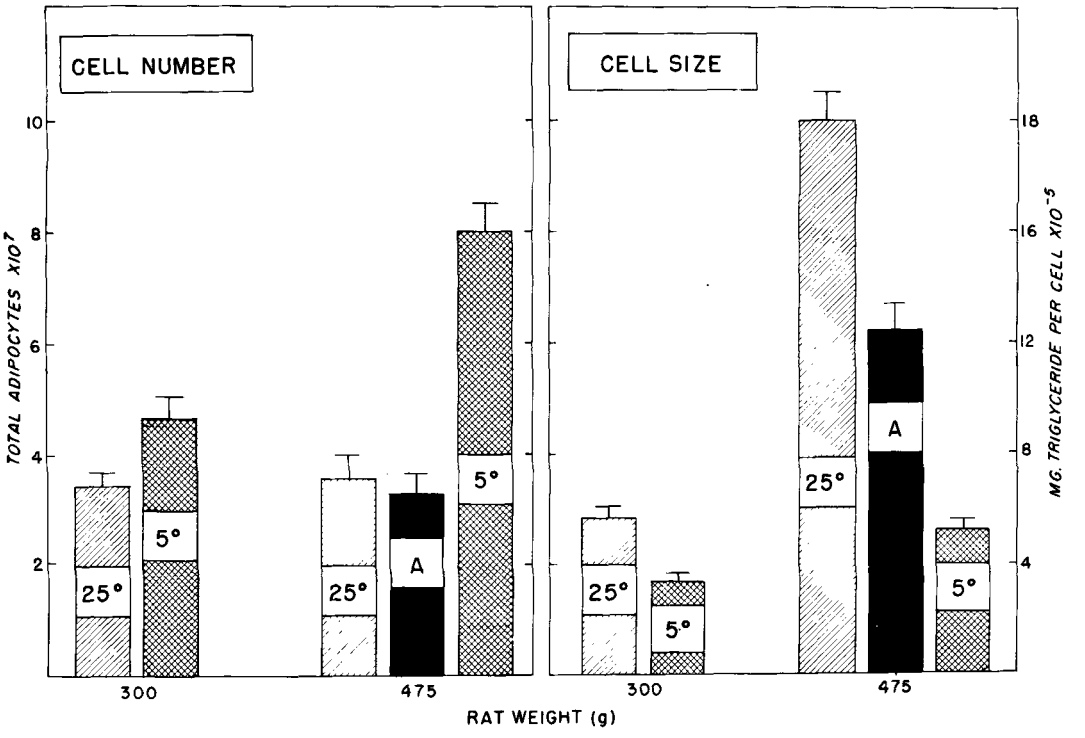

FIG. 3. Epididymal fat cell number and size of rats exposed to cold after reaching the adult stage. A: Rats were maintained at 25 C until they reached a weight of 300 g. They were then placed at 5 C. 25 C: Rats were kept at 25 C throughout the experiment. 5 C: Rats weighing 100 g were placed at 5 C and remained there throughout the experiment.

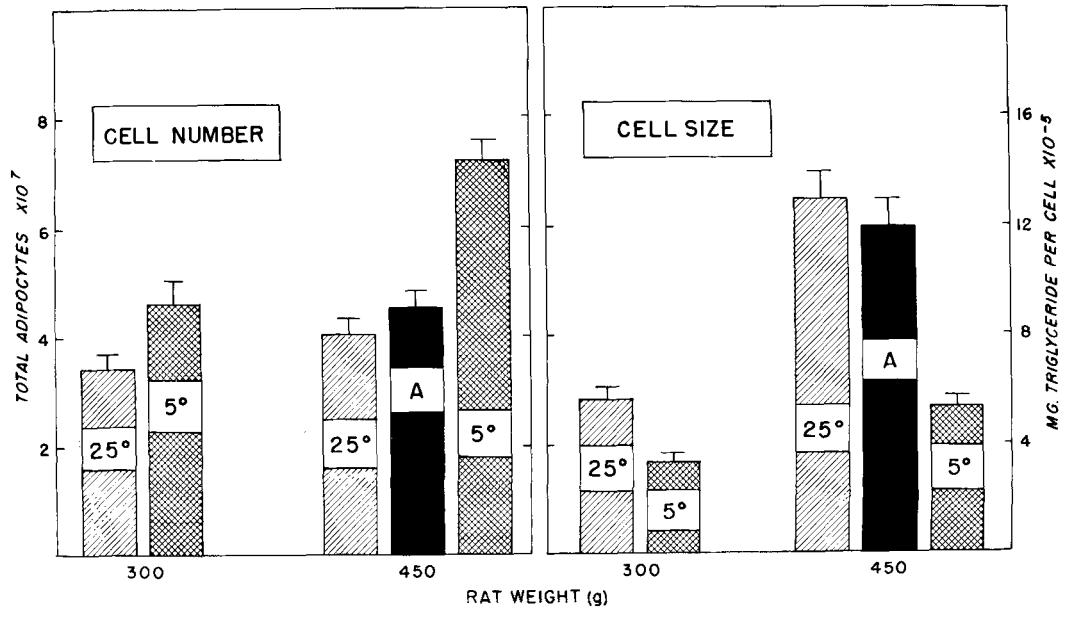

FIG. 4. Epididymal fat cell number and size of rats returned to 25 C after cold exposure. A: Rats were kept at 5 C from 100 g to 300 g. They were then transferred to 25 C. Other groups were as indicated in Figure 3.

TABLE III

Increase in Epididymal Fat Pad Mass and Its Lipid Content in Rats Whose Weight Increased From 300 to 450 g

Temperature	Body weight, g	Pad weight increase, g	Lipid weight increase, g
25 C	454 7[a]	3.35 .30	3.05 .29
5 C	450 6	1.85 .26	1.70 .26
5 C→25 C[b]	449 7	3.92 .45	3.66 .43

[a]Mean ± S.E. There were 12 rats in each group.
[b]Rats were maintained at 5 C from 100 to 300 g. They were then transferred to 25 C.

to hypertrophy rather than hyperplasia (Fig. 3). If in the reverse experiment rats are maintained in the cold during early growth and transferred to a normal ambient temperature after they reach a weight of 300 g, hyperplasia ceases (Fig. 4). The epididymal fat pads grow at a rate equivalent to those from rats maintained from an early age at 25 C (Table III). Just as with rats kept at 25 C from early age, growth of adipose tissue is primarily due to hypertrophy rather than hyperplasia.

DISCUSSION

Adipose tissue triglycerides constitute the major storage form of oxidizable substrate in mammals. The body's reserve of carbohydrate, in the form of glucose and glycogen, are very limited and, except during the interval immediately following meals, it utilizes primarily calories supplied by stored fat. Mobilization of this energy store involves hydrolysis of the triglycerides to FFA and glycerol, which are then released to the extracellular fluid for subsequent transport to the sites of utilization. Since mobilization of fat appears to be regulated by the energy requirements of the animal, it is noteworthy that norepinephrine increases the absolute rate of triglyceride hydrolysis in adipose tissue (7). When the effect of different concentrations of norepinephrine on the release of FFA or glycerol by adipose tissue is measured, a typical dose response curve is obtained (8).

There is considerable evidence that norepinephrine is involved in the process of cold acclimation. Circulatory levels of norepinephrine, as deduced from urine output, are increased in the rat exposed to cold (9-10). Comparison of the in vitro effects of norepinephrine on isolated fat cells, reveals that at all concentrations of norepinephrine, lipolysis is greater in cells from cold acclimated rats (8) than in control animals. Furthermore, both norepinephrine (4-11) and cold exposure (12-13) cause an immediate and marked rise in the metabolic rate with little or no detectable muscle activity. It has also been demonstrated by Hsieh (14) that adrenolytic and ganglionic blocking agents prevent the rise in oxygen consumption that usually accompanies cold exposure of the curarized, cold-acclimated rat and that norepinephrine reverses this effect.

It has been reported by several authors that aging decreases the lipolytic effect of norepinephrine (15-16). In man and in rats, the norepinephrine-induced rise in serum FFA decreases with age (17). It is interesting that the calorigenic effect of norepinephrine also decreases with age. Recent studies by Salans et al. (18) indicate that the cellularity of adipose tissue is of prime importance in evaluating its metabolic activity. Indeed the lipolytic response to norepinephrine in isolated fat cells was found to be closely related to the size of the fat cells (6). The larger the cell size, the less responsive it was to norepinephrine. Since the growth of adipose tissue in the rat occurs primarily by hypertrophy, it is not surprising that the lipolytic effect of norepinephrine decreases with age.

If the calorigenic effect of norepinephrine is dependent on an increased mobilization of FFA from adipose tissue, which makes more FFA available for skeletal muscle to oxidize, animals with smaller fat cells should respond to norepinephrine with a larger calorigenic effect. This in fact is so. The calorigenic effect is greatest in young rats with small fat cells and decreases progressively with age as the fat cells become larger. The accentuated calorigenic effect of norepinephrine in cold-acclimated rats is consistent with this interpretation, since adipose tissue from rats kept in the cold has a much larger number of cells which are much smaller in mass. This conclusion is further supported by the observation that rats exposed to cold, only after they have reached the adult stage, do not develop this accentuated calorigenic response to norepinephrine. Since cell proliferation does not continue in these rats, as it did in rats exposed to cold at an early age, there are fewer

and larger fat cells.

The importance of the lipolytic action of norepinephrine for its calorigenic effect is not clear. Since it is uncertain whether an increase in plasma FFA causes increased oxygen consumption, it is debatable whether the lipolytic effect of norepinephrine can provoke an increase of oxygen consumption. If, however, the oxygen consumption is stimulated by norepinephrine via some other mechanism than its lipotropic action, it is clear that the oxidation of FFA must increase. A stimulation of FFA mobilization may therefore be necessary to furnish substrate if a large increase in oxygen consumption is to be sustained.

It should be pointed out that there is evidence which is not entirely consistent with the hypothesis that the accentuated norepinephrine response in cold-acclimated rats is solely due to adipose tissue cellularity. Isolated fat cells from cold-acclimated animals exhibit an increased sensitivity to norepinephrine which is independent of fat cell size (6). It was noted that norepinephrine-stimulated lipolysis in young rats kept at normal ambient temperature was considerably less than that from rats exposed to cold, even though the fat cell size in adipose tissue from these animals was not appreciably different. It would seem then that the accentuated norepinephrine response in cold-exposed rats is only partially explained by the increased cellularity of adipose tissue. Cold acclimation must involve another adaptation which makes adipose tissue more sensitive to the effects of norepinephrine. It is now well established that the lipolytic response of adipose tissue to norepinephrine is mediated through cyclic 3'-5' AMP, synthesized from ATP by norepinephrine-stimulated adenyl cyclase (19-20). A recent report (21) showing an accentuated effect of norepinephrine on the accumulation of cyclic 3'-5' AMP in isolated fat cells from cold acclimated rats might indicate a second adaptation which results in an increased sensitivity to the effects of norepinephrine in these animals.

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Mass Spectrometric Analysis of Long Chain Alk-1-enyl Ether Esters and Alkyl Ether Esters of Diols[1]

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ABSTRACT

The mass spectra of homologous series of long chain alk-1-enyl ether esters and alkyl ether esters of short chain diols were determined, and general patterns of fragmentation were established. Both types of diol lipids yielded ions characteristic of the alkoxy or alk-1-enyloxy moiety and the acyl moiety, as well as ions indicative of the constituent short chain diol. Prominent ions were formed from both types of ether esters due to the loss of the alkoxy or alk-1-enyloxy moieties giving rise to ions for which cyclic structures are proposed. High resolution mass spectrometry and deuterium labeling techniques were used to verify the composition of ions and to substantiate fragmentation mechanisms.

INTRODUCTION

Esters (2-4), diethers (5), and alk-1-enyl ether esters (4) of short chain dihydric alcohols occur as minor lipid constituents in animals, plants and microorganisms. Alkyl ether esters of diols have not been detected in nature (6). Studies of the chromatographic behavior and other physical characteristics of these lipids (7-11) revealed the great similarity of diol lipids to the more abundant esters, ethers and ether esters derived from glycerol. Mass spectrometry is a promising tool for detection and analysis of naturally occurring diol lipids (12).

Mass spectral fragmentation patterns of long chain esters (13-15), such as methyl esters (16-21), wax esters (18,22,23), diol esters (12) and triglycerides (13,24-27) have been studied thoroughly. Less is known about the electron impact induced formation of ions from long chain alk-1-enyl ethers (11,28,29) and alkyl ethers (13,30-34).

We describe here the mass spectra of alk-1-enyl ether esters and alkyl ether esters of short chain diols. Mass spectrometric data of homologous series of these compounds are reported and general fragmentation patterns are established. To ascertain the composition and structure of fragments, high resolution mass spectrometry and deuterium labeling in specific positions were used. Mass spectrometry is shown to be a means of identifying diol ether esters, their constituent short chain diols, and their long chain moieties.

MATERIALS AND METHODS

Alk-1-enyl ether esters of ethanediol (2-alk-1'-enyloxy-1-*O*-acyl-ethanols) (11) and alkyl ether esters of ethanediol (2-alkoxy-1-*O*-acyl-ethanols) and of 1,3-propanediol (3-alkoxy-1-*O*-acyl-propanols) (7) were prepared as described previously. The alk-1-enyl ether ester of 1,1,2,2-tetradeuteroethanediol was synthesized in 96% isotopic purity using deuterated glycol International Chemical and Nuclear Corp.) in the transacetalation step of the synthesis (10). The alkyl ether ester of 1,1,2,2-tetradeuteroethanediol (95% isotopic purity) was obtained through hydrogenation of the corresponding alk-1-enyl ether esters using platinum dioxide as catalyst and hexane as solvent. 1,2-Di-*O*-hexadecanoyl-1,1,2,2-tetradeuteroethanediol (97% isotopic purity) was prepared by heating deuterated glycol with hexadecanoic acid under reduced pressure (15 mm) with *p*-toluenesulfonic acid (27), and the product was purified by adsorption thin layer chromatography. All compounds were pure as judged by adsorption chromatography, reversed-phase partition chromatography and gas chromatography.

Low resolution mass spectra were recorded on a Hitachi Perkin-Elmer RMU-6D single focusing, magnetic scanning instrument at 70 and 15 eV. The samples were introduced through the direct insertion system at a temperature (110-300 C) at which a constant total ion concentration was achieved. Simultaneous scanning of perfluorokerosene, which was introduced through the liquid inlet system, permitted accurate counting of peaks. Abundances of ions are given relative to the most prominent peak. Ranges of abundances refer to saturated compounds only. High resolution mass spectra were recorded on an AEI MS-9 instrument at the Molecular Structure Laboratory, Department of Chemistry, Purdue University, Lafayette, Indiana.

[1]This is part VII in the series "Naturally Occurring Diol Lipids" (part VI is Reference 1) and part VIII in the series "Mass Spectrometry of Lipids" (part VII is Reference 23).

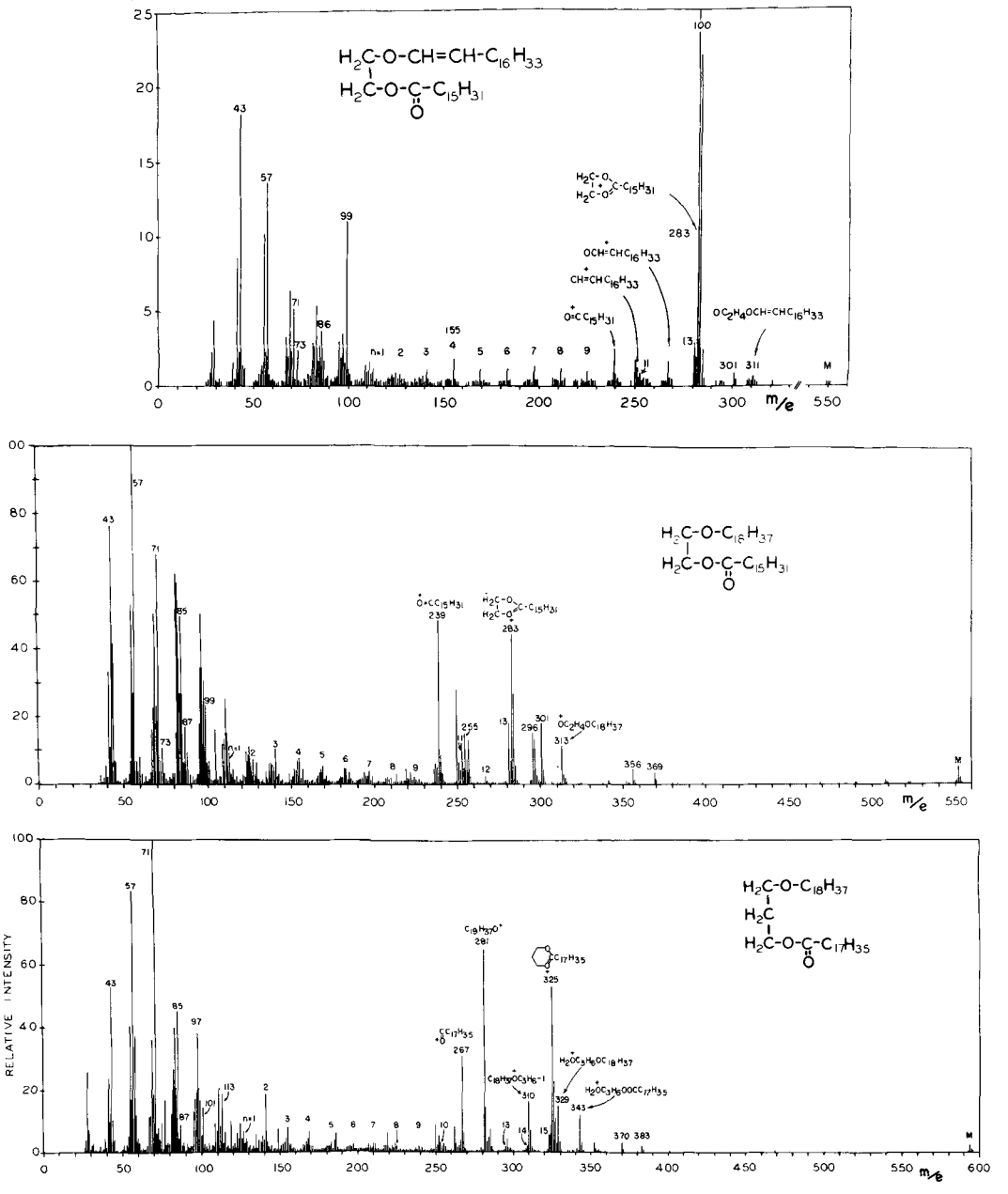


FIG. 1. Mass spectra of 2-octadec-1'-enyloxy-1-*O*-hexadecanoyl-ethanol (top), 2-octadecyloxy-1-*O*-hexadecanoyl-ethanol (center), and 3-octadecyloxy-1-*O*-octadecanoyl-propanol (bottom) recorded at 70 eV.

RESULTS AND DISCUSSION

Mass spectra of alk-1-enyl ether esters and alkyl ether esters of short chain diols recorded at low ionization potentials indicated the major modes of fragmentation. They were helpful in interpreting the more reproducible and more complex spectra measured at 70 eV. Only the latter spectra are being discussed. In the spectra of all alk-1-enyl ether esters and alkyl ether esters of diols several series of peaks are present which are correlated with hydrocarbon ions. These series are m/e 43+14x (C_yH_{2y+1}), m/e 42+14x (C_yH_{2y}), m/e 41+14x (C_yH_{2y-1}), m/e 40+14x (C_yH_{2y-2}), m/e 39+14x (C_yH_{2y-3}) and m/e 38+14x (C_yH_{2y-4}). High resolution

TABLE I

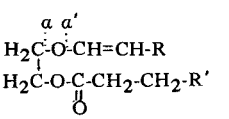
Relative Intensities of the Principal Ions in the Mass Spectra of 2-Octadec-1′-enyloxy-1-*O*-Acyl-Ethanols

	$H_2COCH{=}CHC_{16}H_{33}$ $H_2COOCC_nH_{2n+1}$	n=13		n=15		n=17		Δ8-17:1		$D_2COCH{=}CHC_{16}H_{33}$ $D_2COOCC_{15}H_{31}$	
Ion	Ion structure	m/e	RI	m/e	RI	m/e	RI	m/e	RI	m/e	RI
	C_3H_7	43	21		18		18		18	43	13
	C_2H_5O	45	1.3		1.2		1.1		1.3	49	0.5
	C_4H_9	57	15		14		15		12	57	9.6
XXV	$C_2H_4O_2$	60	0.5		0.5		0.8		0.5	60	0.5
XXVI	$C_2H_5O_2$	61	0.4		0.4		0.6		0.4	61	0.3
	$C_2H_7O_2$	63	0.3		0.3		0.4		0.4	67	2.3
	C_5H_{11}	71	5.8		5.2		5.2		4.6	71	4.2
XIV	$C_3H_5O_2$	73	2.3		2.4		2.2		2.4	77	1.5
	C_6H_{13}	85	2.9		2.7		3.2		2.3	85	2.3
XV	$C_4H_6O_2$	86	4.4		3.7		4.2		3.6	90	3.5
XVI	$C_4H_7O_2$	87	1.8		1.7		1.8		1.3	91	2.3
XVII	$C_5H_7O_2$	99	10		11		10		16	103	8.3
XXVII	C_4H_9O	105	0.3		0.4		0.5		0.9	109	1.1
	$C_5H_7O_2C_4H_8$	117	0.3		0.4		0.4		0.4	121	0.5
	$C_5H_7O_2C_4H_8$	155	2.1		1.8		2.5		5.4	159	1.6
VI	$C_{18}H_{34}$	250	1.7		1.7		2.0		1.1	250	1.6
III	$C_{18}H_{35}$	251	1.1		1.2		1.3		1.0	251	1.2
II	$C_{18}H_{35}O$	267	1.3		1.6		3.8		1.0	267	1.6
IV	$C_{18}H_{35}OH$	268	0.4		0.5		1.2		0.4	268	0.4
XIII	$C_{18}H_{35}OC_2H_4O$	311	0.7		0.6		100		5.7	315	0.6
IX	$C_nH_{2n+1}CO$	211	3.1	239	2.4	267	3.8	265	2.2	239	1.4
X	$C_nH_{2n+1}COO$	227	0.3	255	0.5	283	0.7	281	0.4	255	0.2
XI	$C_nH_{2n+1}COOH$	228	0.3	256	0.4	284	0.6	282	0.4	256	0.3
XII	$C_nH_{2n+1}COOH_2$	229	0.4	257	0.4	285	0.5	283	0.4	257	0.5
VIII	$C_nH_{2n-1}COOC_2H_4$	253	2.3	281	2.8	309	2.9	307	2.3	285	2.5
VII	$C_nH_{2n}COOC_2H_4$	254	1.4	282	1.9	310	2.0	308	11	286	5.0
I	$C_nH_{2n+1}COOC_2H_4$	255	100	283	100	311	100	309	100	287	100
I	(% of total ionization)		(27.9)		(28.4)		(26.6)		(19.6)		(33.1)
V	$C_nH_{2n+1}COOC_2H_4OH_2$	273	0.9	301	0.8	329	0.9	327	0.5	305	0.8
M		522	0.3	550	0.2	578	0.3	576	0.7	554	0.2

mass spectrometry showed that in the series 43+14x ions $C_yH_{2y-1}O$ and $C_zH_{2z-3}O_2$ contribute significantly to the peak intensities. Series 42+14x contains $C_yH_{2y-2}O$. The hydrocarbon fragments are usually of low molecular weight and of little diagnostic value, even though one of the ions, m/e 57, is the base peak in the spectra of alkyl ether esters.

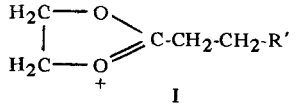
Alk-1-enyl Ether Esters of Ethanediol

All *cis* and *trans* isomers of alk-1-enyl ether esters of ethanediol (2-alk-1′-enyloxy-1-*O*-acylethanols) exhibited identical mass spectra, similar to what is known of unsaturated fatty acid



methyl esters (19). The spectra showed small but recognizable molecular ion peaks (Fig. 1). An ion $[M-18]^+$ which is a significant fragment formed from triglycerides (24,27) and diol esters (12,27) is also produced from 1,2-di-*O*-hexadecanoyl-1,1,2,2-tetradeuteroethanediol, but not from alk-1-enyl ether esters, indicating the hydrogens lost do not originate from the ethanediol moiety, but rather from methylene groups of a pair of acyl moieties.

Ether Cleavages. The major mode of fragmentation is cleavage α to the ether oxygen. Loss of the neutral alkenyloxy moiety RCH=CHO, forms ion I which is a distinct characteristic of these spectra (Table I). Ion I contains the intact diol fragment because it is shifted four mass units for the perdeuterated glycol analog. A cyclic structure was suggested for this ion (11). The ion



I

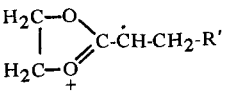
$[M-RCOO]^+$ formed in high abundance from diol diesters (12) is identical to the cyclic ion I.

Cleavage in the α position also produced ions with charge retention on the alk-1-enyloxy group as was observed for alkyl vinyl ethers (35). Ion II, $[RCH=CHO]^+$ (1.3-1.6%) is less abundant when the acyl group is unsaturated. With 2-octadec-1′-enyloxy-1-*O*-octadecanoyl-ethanol (Table I) fragment IX coincides with ion II, contributing to the higher intensity of m/e 267. Alternatively, ether cleavage at position α′ yields $[RCH=CH]^+$ (III) (1.1-1.3%), whereas complementary ions $[R'CH_2CH_2COOCH_2CH_2O]^+$ are not observed.

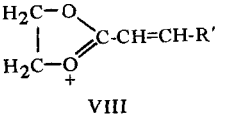
Alcohol fragments are produced from alk-1-enyl ether esters of ethanediol only to a small extent. Ion $[RCH=CHOH]^+$ (IV) occurs up to 1.2%, whereas $[R'CH_2CH_2COOCH_2CH_2OH]^+$ is absent. Such alcohol fragments are formed in much higher abundance from simple aliphatic ethers of the type R-O-R (30-33) where competitive modes of fragmentation, such as that leading to ion I, cannot take place.

An ion $[R'CH_2CH_2COOCH_2CH_2OH_2]^+$ (V) is found in all spectra of alk-1-enyl ether esters examined. Formation of a protonated alcohol involves α′-fragmentation and, presumably, double hydrogen rearrangement in which the transferred hydrogens may originate from positions 4 and 5 of the alk-1-enyl moiety in analogy to the fragmentation of dialkyl ethers (33). The corresponding ion $[RCH=CH-OH_2]^+$, which would be produced by α cleavage, requires hydrogen transfer from the position occupied by the carbonyl group. Hence, the presence of ion V and the absence of $[RCH=CH-OH_2]^+$ support the proposed mechanism of a double hydrogen rearrangement (33). Loss of water from ion V may be one of the pathways leading to the cyclic ion I; however, peaks for metastable ions were not observed.

Formal loss of glycol monoester from the alk-1-enyl ether esters with charge retention on the alk-1-enyl residue (30) leads to ion VI, $[RCH=CH-1]^+$ (1.7-2.0%). Formal loss of alkenol yields ion VII (1.4-2.0%), and loss of an additional hydrogen yields ion VIII (2.3-2.9%). In the spectrum of deuterated alk-1-enyl



VII



VIII

ether ester (Table I) ions VII and VIII were shifted by four mass units, as was observed for ion I, but ions having 3 or 4 mass units less than ion I were not observed. The apparent increase in the intensity of ion VII was due to contribution of ion I formed from deuterated alk-1-enyl ether ester of lesser deuterium content, whereas ion VIII was of almost equal intensity in the spectra of the deuterated and nondeuterated compounds. Therefore, the hydrogens lost in the formation of ions VII and VIII do not arise from the glycol moiety, but must arise from the acyl chain. Enolization of the ester group followed by elimination of the alk-1-enyloxy group and the enol hydrogen, or simple loss of hydrogen from cyclic ion I, could account for formation of ion VII. In the spectra of unsaturated esters of alk-1-enyl glycol ethers, ion VII becomes a major fragment (11%), whereas ion VIII remains at an intensity equal to that for

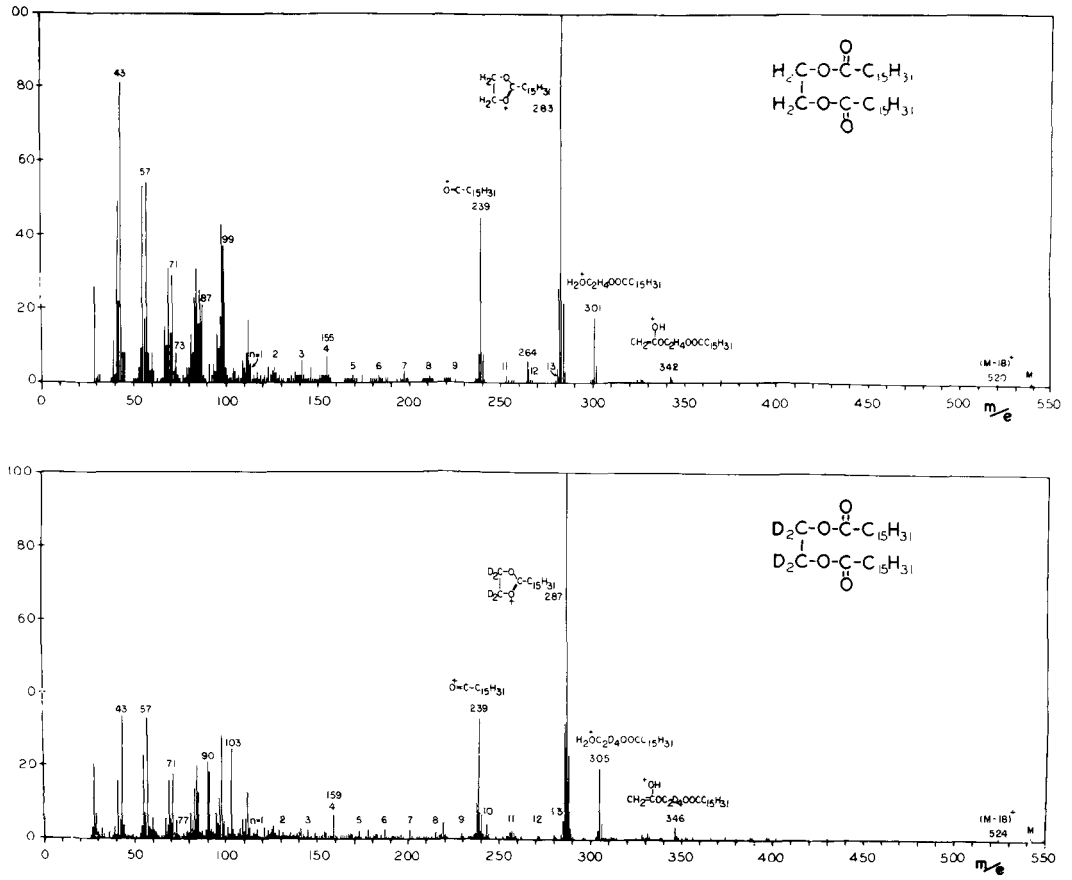
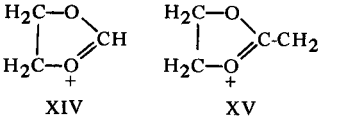


FIG. 2. Mass spectra of 1,2-di-*O*-hexadecanoyl-ethanediol and 1,2-di-*O*-hexadecanoyl-1,1,2,2-tetradeuteroethanediol recorded at 70 eV.

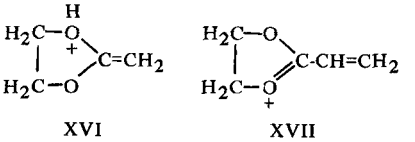
saturated alk-1-enyl ether esters.

Ester Cleavages. The acylium ion, $[R'CH_2CH_2CO]^+$ (IX), commonly formed from long chain esters, is produced from alk-1-enyl ether esters in 2.4-3.8% relative abundance. Peaks of lower intensities are caused by ions $[R'CH_2CH_2COO]^+$ (X) 0.3-0.7%, $[R'CH_2CH_2COOH]^+$ (XI) 0.3-0.6%, and $[R'CH_2CH_2COOH_2]^+$ (XII) 0.4-0.5% (15). An ion $[RCH{=}CHOCH_2CH_2O]^+$ (XIII), formed by elimination of an acyl residue, is present in low abundance. A cyclic structure for XIII, similar to that postulated for an ion derived from alk-1-enyl glycerol ethers (28) is plausible. However, the major cyclic ions produced from alk-1-enyl ether esters contain the acyl skeleton rather than the alk-1-enyl residue.

Deuterium labeling in the ethanediol moiety shifted ion m/e 73 (XIV) to 77, m/e 86 (XV) to 90, m/e 87 (XVI) to 91, m/e 99 (XVII) to 103, and the homologous series 99+14n to 103+14n,



XIV XV



XVI XVII

demonstrating the presence of the glycol moiety in these ions. Ions XIV-XVII may arise through cleavage of the bonds α, β, γ, etc., to the ester function (13,20), loss of the alk-1-enyloxy residue and cyclization. α-Cleavage of the ester gives rise to m/e 73 (XIV) which probably has a 1,3-dioxolane skeleton. An ion m/e 73 was previously observed in the spectra of long chain esters of diols (12), and a structure, $[CH_2{=}COH\text{-}OCH_2]^+$, was tentatively

TABLE II

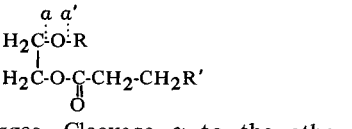
Relative Intensities of the Principal Ions in the Mass Spectra of 2-Alkoxy-1-*O*-Acyl-Ethanols

	$H_2COC_mH_{2m+1}$ / H_2C-$OOCC_nH_{2n+1}$	m=14 n=15		m=16 n=15		m=16 n=17		m=18 n=15		m=18 n=17		Δ9-18:1 Δ8-17:1		$D_2COC_{18}H_{37}$ / $D_2COOCC_{15}H_{31}$	
Ion	Ion structure	m/e	RI	m/e	RI	m/e	RI	m/e	RI	m/e	RI	m/e	RI	m/e	RI
	C_3H_7	43	72		82		68		76		71		52	43	87
	C_2H_5O	45	7.0		11		5.6		6.9		7.5		6.4	49	4.6
	C_4H_9	57	100		100		100		100		100		49	57	100
XXV	$C_2H_4O_2$	60	5.1		4.9		7.3		8.0		6.0		5.5	60	4.1
XXVI	$C_2H_5O_2$	61	2.4		2.4		3.4		3.2		3.5		2.4	61	3.6
	$C_2H_7O_2$	63	2.5		2.2		2.2		2.7		3.4		3.3	67	15
	C_5H_{11}	71	69		61		70		68		72		24	71	60
XIV	$C_3H_5O_2$	73	7.7		7.2		11		11		9.8		6.7	77	1.5
	C_6H_{13}	85	52		44		51		50		56		15	85	40
XV	$C_4H_6O_2$	86	6.8		5.2		6.1		6.3		7.0		4.8	90	4.4
XVI	$C_4H_7O_2$	87	16		15		15		17		19		5.2	91	13
XVII	$C_5H_7O_2$	99	25		20		26		24		27		20	103	13
XXVII	$C_4H_9O_3$	105	22		14		17		16		18		5.5	109	22
	$C_5H_9O_3$	117	2.0		1.5		1.8		2.3		2.5		3.3	121	2.9
	$C_5H_7O_2C_4H_8$	155	7.2		5.8		10		7.8		11		12	159	2.2
XXIII	$C_{m-2}H_{2m-4}$	168	5.1	196	2.5		3.3	224	2.2		3.1	222	4.2	224	1.8
VI	C_mH_{2m-2}	194	38	222	27		31	250	28		31	248	7.0	250	6.9(251,7.8)
XXII	C_mH_{2m}	196	6.6	224	3.9		5.9	252	4.2		4.0	250	16	252	7.0
XVIII	C_mH_{2m+1}	197	18	225	12		20	253	13		18	251	4.8	253	8.1
XIX	$C_mH_{2m+1}O$	213	2.7	241	2.2		3.4	269	0.6		3.1	267	4.2	269	0.4
XXI	$C_{m+1}H_{2m+1}O$	225	21	253	13		18	281	18		19	279	3.0	282	4.0(283,3.4; 284,2.5)
XXX	$C_mH_{2m+1}OC_2H_4$-1	240	28	268	14		25	296	16		18	294	2.4	300	8.6(299,11)
XXIX	$C_mH_{2m+1}OC_2H_4$	241	24	269	15		22	297	14		17	295	2.4	301	2.3
XXXI	$C_mH_{2m+1}OC_2H_4O$	257	26	285	16		28	313	12		23	311	14	317	12
XXIV	$C_mH_{2m+1}OC_2H_4OC_2H_3O$	300	5.1	328	4.0		5.3	356	4.5		5.0	354	1.8	360	4.4
XXVIII	$C_mH_{2m+1}OC_2H_4OC_3H_4O$	313	5.3	341	3.1		5.3	369	3.6		4.9	367	1.5	373	3.2
IX	$C_nH_{2n+1}CO$	239	56		38	267	48	239	49	267	56	265	39	239	39
X	$C_nH_{2n+1}COO$	255	16		13	283	17	255	16	283	19	281	3.0	255	13
XI	$C_nH_{2n+1}COOH$	256	7.0		5.8	284	11	256	7.7	284	9.8	282	2.1	256	5.5
XII	$C_nH_{2n+1}COOH_2$	257	26		8.3	285	28	257	14	285	13	283	2.4	257	4.7
VIII	$C_nH_{2n-1}COOC_2H_4$	281	4.2		3.4	309	4.9	281	18	309	5.6	307	4.2	285	4.8
VII	$C_nH_{2n}COOC_2H_4$	282	4.7		3.7	310	5.5	282	7.3	310	6.4	308	16	286	5.3
I	$C_nH_{2n+1}COOC_2H_4$	283	42		31	311	42	283	45	311	53	309	100	287	43
I	(% of total ionization)		(2.3)		(2.0)		(2.3)		(2.4)		(2.6)		(4.3)		(2.8)
XX	$C_nH_{2n+1}COOC_2H_4O$	299	0.5		0.5	327	0.6	299	1.2	327	0.8	325	2.4	303	0.7
V	$C_nH_{2n+1}COOC_2H_4OH_2$	301	18		14	329	17	301	18	329	19	327	6.4	305	18
M		496	4.8	524	6.0	552	5.8	552	5.2	580	4.5	576	10	556	6.5
Metastable peaks															
V → IX		190.2		188.9		216.7		190.3		215.9		None		187.1	
	(calc.)		(189.8)		(189.8)		(216.7)		(189.8)		(216.7)				(187.3)
XXX → XVIII		161.2		188.9		188.7		216.1		215.9				213.3	
	(calc.)		(161.7)		(188.9)		(188.9)		(216.2)		(216.2)				(213.4) (214.1)

assigned to this ion. As ion m/e 73 is shifted to m/e 77 in the spectra of alk-1-enyl ether esters (Table I) and to a lesser degree also in the spectra of diesters of deuterated ethanediol (Fig. 2), and because ion m/e 75 is virtually absent, the cyclic ion XIV probably contributes to the intensities of m/e 73 in both diol lipids. McLafferty rearrangement involving β-cleavage of the acyl chain leads to ion XVI, which on further loss of one hydrogen gives ion XV. The high abundance of ion XVII can be explained by an energetically favored allylic homolytic cleavage. Deuterium labeling showed that the ions of the homologous series 99+14n are not part of the series C_yH_{2y+1}. Ion m/e 155 of the series 99+14n (n=4) and the parent member of the series are particularly abundant. Unsaturation enhances the intensities in this series.

Alkyl Ether Esters of Ethanediol

The mass spectra of alkyl ether esters of ethanediol (2-alkoxy-1-*O*-acyl-ethanols) exhibit peaks corresponding to M^+, and also $[M+1]^+$ and $[M+2]^+$ (Fig. 1). Ions due to the loss of water, $[M-18]^+$, are absent. The hydrocarbon fragment $[C_4H_9]^+$, m/e 57, is the base peak.



Ether Cleavages. Cleavage α to the ether oxygen with charge retention on the acyloxyethyl fragment yields ion I (31-53%) (Table II). The alkyl ion R^+ (XVIII) produced through α'-cleavage is present (12-20%). Charge retention on the oxygen-containing fragments is less favored giving considerably weaker ions $[RO]^+$ (XIX) (0.6-3.4%), and $[R'CH_2CH_2COOCH_2CH_2O]^+$ (XX) (0.5-1.2%). Elemental composition of these ions was confirmed by high resolution mass spectrometry.

From alkyl ether esters of ethanediol an ion XXI (13-21%) is formed which formally contains the alkoxy residue plus one carbon atom. Precise mass measurements confirmed its elemental composition as $C_{m+1}H_{2m+1}O$. The deuterated alkyl ether ester (Table II) yielded ions of type XXI at m/e 282, 283 and 284 containing one, two or three deuteriums, respectively. The structure of XXI is not known. Ion XXI superimposes upon ion VIII when the alkyl chain is two methylene groups longer than the acyl chain.

Formal loss of glycol monoester gives ion $[R-1]^+$ (XXII) (3.9-6.6%) and further loss of an ethylene group (33) yields ion $[R-C_2H_5]^+$ (XXIII) (2.2-5.1%). Elimination of an alcohol from the alkyl ether ester yields the cyclic ion VII (4.7-7.3%). Additional loss of hydrogen leads to ion VIII (3.4-18%). High resolution mass spectrometry confirmed the elemental compositions of ions VII and VIII, and deuteration of the ethanediol moiety shifted ions I, VII and VIII four mass units.

An abundant ion VI (27-33%) is observed in the spectra of alkyl ether esters at $[R-3]^+$, whose elemental composition is C_mH_{2m-2}. However, the spectrum of the alkyl ether ester of the deuterated ethanediol indicated the presence of as many as two deuterium atoms in ions VI. Ions VI formed from alk-1-enyl ether esters occur in much lower abundances, and in the case of the deuterated analogue, fragment VI does not contain deuterium. Ions similar to VI are also produced from dialkyl ethers of diols (34) and to a small extent from dialkyl ethers (33). Monoalkyl ethers of diols (34) and diesters of diols (12) do not form this ion.

α'-Cleavage and double hydrogen rearrangement (33) yield ion V (14-19%). The position of the ester function relative to the ether grouping prohibits formation of $[ROH_2]^+$, as was the case with the alk-1-enyl analog. Loss of water from V may lead to I, but the corresponding metastable ion was not observed. Ion V can lose the glycol fragment m/e 62 ($C_2H_6O_2$) to give acylium ion IX, as was substantiated by metastable ions m/e 190.2 (calc. m/e 189.9) and m/e 216.7 (calc. m/e 216.7) from the hexadecanoates and octadecanoates, respectively. In the spectrum of the deuterated ether ester the metastable ion occurs at m/e 187.1 (calc. m/e 187.3).

Ester Cleavages. The acylium ion (IX) derived from alkyl ethers esters of ethanediol is very intense (38-56%). Significant peaks are also observed for ions having one, two or three hydrogens less than the acylium ion IX (4.2-8.7%, 4.5-8.7% and 3.6-6.6%, respectively) (Fig. 1). Precise mass measurements verified the compositions of these ions. In addition, acyloxy ions X (13-19%), "acid" ions XI (5.8-11%), and ions $[R'CH_2CH_2COOH_2]^+$ XII (8.3-28%) are observed (Table II). Ion XI is probably formed via McLafferty rearrangement, and formation of ion XII involves a double hydrogen transfer.

The McLafferty rearrangement with loss of most of the chain from the acyl moiety yields ion $[ROCH_2CH_2OC(OH)=CH_2]^+$ (XXIV) (1.1-5.3%); the elemental composition of XXIV was confirmed. Ion XXIV may be cleaved involving one or two hydrogen transfers to give ion XXV m/e 60, $[CH_2=C(OH)_2]^+$ (4.9-8.0%), or ion XXVI m/e 61 $[CH_2=C(OH)OH_2]^+$ (2.4-3.5%), respectively. Following the double hydrogen rearrangement ion XXIV may alterna-

tively yield ion m/e 105 $[H_2OCH_2CH_2OC(OH)=CH_2]^+$ (XXVII) (14-22%). Deuterium labeling demonstrated that ions XXIV and XXVII include the ethanediol moiety (Table II). Elimination of the alkoxy moiety from ion XXIV and cyclization leads to ion m/e 87 (XVI) (15-19%).

3,4-Cleavage of the acyl moiety results in a weak ion XXVIII $[ROCH_2CH_2OCOCH_2CH_2]^+$ (3.1-5.3%). Formal loss of alkanol from XXVIII yields the more abundant ion m/e 99 (XVII) (20-27%). Deacyloxylation gives ion $[ROCH_2CH_2]^+$ XXIX (14-24%), loss of long chain fatty acid leads to $[ROCH_2CH_2\text{-}1]^+$ XXX (14-28%), and deacylation gives $[ROCH_2CH_2O]^+$, XXXI (12-26%). The compositions of these ions were confirmed by deuterium labeling and high resolution mass spectrometry. Formation of ion XXX involves equal loss of hydrogen from the alkoxy moiety or from the ethanediol residue as was shown by deuterium labeling. Ion XXX loses the diol fragment m/e 43 (C_2H_3O) to yield the alkyl ion XVIII. This transition is evident from metastable ions m/e 161.2 (calc. m/e 161.7), m/e 188.9 (calc. m/e 188.9) and m/e 216.1 (calc. m/e 216.2) in the spectra of the tetradecyl, hexadecyl and octadecyl ethers, respectively. In the spectrum of the deuterated ether ester a broad peak occurs at m/e 213-214 indicating metastable ions m/e 213.4 and 214.1 arising from m/e 300 and 299, respectively. When the acyl and alkyl moieties are identical in chain length, these metastable ions coincide with the metastable ions for the transition of ion V to ion IX (Table II).

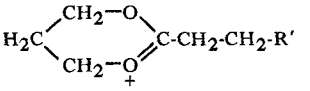
In mass spectra of alkyl ether esters of ethanediol, peaks are found at m/e 73 (XIV), 86 (XV), 87 (XVI), 99 (XVII) and 99+14n (Fig. 1), similar to those in spectra of alk-1-enyl ether esters. Corresponding peaks in the spectrum of the deuterated ethanediol ether ester were shifted by four mass units. High resolution mass spectrometry showed that these ions are not homogeneous. Deuterium labeling and high resolution mass spectrometry also indicated that ions m/e 45 $[CH_2CH_2OH]^+$, 63 $[HOCH_2CH_2OH_2]^+$, and 117 $[HOCH_2CH_2OOCCH_2CH_2]^+$ include the ethanediol moiety. These ions are of diagnostic value for alkyl ether esters of ethanediol because they are not formed from alk-1-enyl ether esters of ethanediol.

Alkyl Ether Esters of 1,3-Propanediol

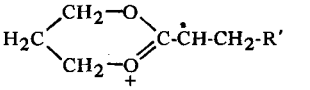
Many ions in the mass spectra of alkyl ether esters of 1,3-propanediol are identical to those formed from ether esters of ethanediol. Other ions containing the 1,3-propanediol moiety are homologous to those derived from ethanediol ether esters, and thus, are shifted by 14 mass units. In mass spectra of propanediol ether esters, the molecular ion peak is present. Ion $[M-18]^+$ is not observed (Fig. 1). In the spectra of saturated compounds, m/e 71 is the base peak, whereas m/e 57 is the most abundant ion formed from unsaturated ether esters of 1,3-propanediol.

Ions identical to those formed from ethanediol derivatives which indicate the ether group, are $[R\text{-}1]^+$ (XXII), R^+ (XVIII), $[RO]^+$ (XIX), and XXI. Those indicating the ester group are $[R'CH_2CH_2CO]^+$ (IX), $[R'CH_2CH_2COO]^+$ (X), $[R'CH_2CH_2COOH]^+$ (XI) and $[R'CH_2CH_2COOH_2]^+$ (XII). Their intensities are similar to those of ethanidol derivatives except for ion XXI which occurs in an abundance of 41-65%.

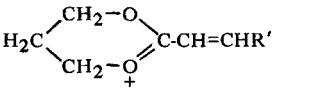
Homologous ions formed from ether esters of 1,3-propanediol are of greater diagnostic value. A major ion is ion I' (54-63%), and in analogy to ion I, a 1,3-dioxane-type structure is proposed. Ions VII' (6%) and VIII' (9%) have one and two mass unist less than ion I', respectively. When the acyl chain is unsaturated, ion VIII' becomes a major ion.



I'



VII'

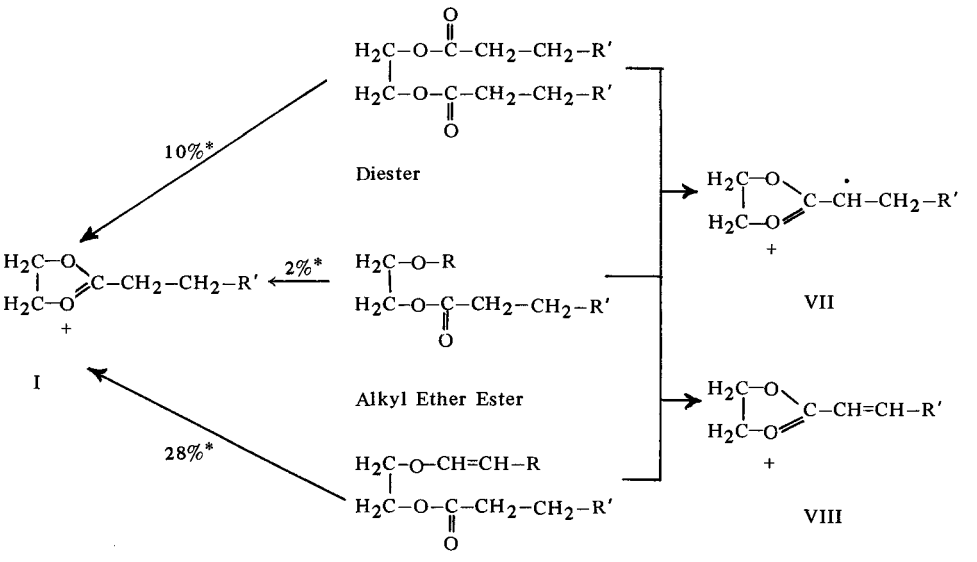


VIII'

Ions formed by deacyloxylation (XXIX'), loss of long chain fatty acids (XXX'), and deacylation (XXXI') also occur. An intense ion having two mass units more than ion XXXI' may have the structure $[ROCH_2CH_2CH_2OH_2]^+$, XXXII'. α'-Cleavage and double hydrogen rearrangement yield ion VI', but $[ROH_2]^+$ is absent. Low mass ions containing the propanediol moiety are m/e 59, 77 and 131, corresponding to ions m/e 45, 63 and 117 produced from ethanediol ether esters.

Conclusion

Neutral diol lipids containing at least one ester function form a common, major fragment of the type $[RCOO(CH_2)_n]^+$. This



Scheme I

ion (I) forms the base peak in the spectra of alk-1-enyl ether esters and also of symmetrical diesters of short chain diols. In the spectra of alkyl ether esters ion I is less prominent (20-30%). Ions I are formed from alk-1-enyl ether esters, symmetrical diesters, and alkyl ether esters in an approximate ratio of 28:10:2, when abundances are expressed as per cent of total ionization. Loss of the alk-1-enyloxy group is highly favored over loss of the alkoxy group. Prevalence of ion I suggests energetically favored structures, such as those of cyclic acetals (Scheme 1).

These ions were chosen to quantitatively determine the composition of a synthetic mixture of 2-octadecyloxy-1-*O*-octadecanoyl-ethanol (52.1 mole %) and 3-octadecyloxy-1-*O*-octadecanoyl-propanol (47.9 mole %). The intensities of ions I, m/e 311 (27.1) and I', m/e 325 (24.3) were in the same proportion as the compositions (52.7% and 47.3%).

Formation of ions VII and VIII probably takes place via enolization of the ester function, followed by expulsion of the alk-1-enyloxy, the second acyloxy, or the alkoxy group, with accompanying loss of the enol hydrogen. Further loss of a hydrogen from the acyl chain leads to a stable allylic structure, ion VIII. Ions m/e 73, 86, 87, 99 and 99+14n occur in the spectra of all three classes of ethanediol-derived lipids. However, the intensties of these ions decline in the order diesters>alkyl ether esters>alk-1-enyl ether esters, unlike the per cent of total ionization for ion I. Hence, these ions are probably not formed from ion I, but may be produced by fragmentation within the acyl chain and loss of the alk-1-enyloxy, alkoxy or acyloxy grouping.

In the spectra of alkyl ether esters the acylium ion is particularly intense similar to what is found for diol diesters. In the case of the alk-1-enyl ether esters, the acylium ion is less abundant due to the favored formation of I. Knowing the acyl function permits identification of the chain length of the diol moiety by comparison with ion I. The ratio of intensities for ions I and IX is characteristic for each class of diol lipids.

Many of the usual fragmentations characteristic for ethers do not occur with long chain ether esters of diols, e.g., water loss with concurrent loss of up to three methylene groups (30), formation of an oxiranium ion (33), and cleavage β to the double bond of the alk-1-enyl ethers (29). This may well be the consequence of the highly favored formation of ion I from the ester portion of these lipids.

Each of the alk-1-enyl ether esters and alkyl ether esters of diols can be identified by its mass spectrum, with the exception of *cis-trans* isomers. Ions characteristic and useful for the identification of the acyl moieties are $[R'CH_2CH_2CO]^+$ (IX), $[R'CH_2CH_2COO]^+$ (X), $[R'CH_2CH_2COOH]^+$ (XI), and

$[R'CH_2CH_2COOH_2]^+$ (XII). The alk-1-enyl and alkyl groups are indicated by several ions of somewhat lower abundance. The ions are $[R-C_2H_5]^+$ (XXIII), $[R-1]^+$ (VI and XXII), $[R]^+$ (III and XVIII), $[RO]^+$ (II and XIX), and XXI. In order to confirm the identity of the diol residue, ions $[RO(CH_2)_n]^+$ (XXIX), $[RO(CH_2)_nO]^+$ (XIII and XXXI), $[RO(CH_2)_n OH]^+$ (XXXII), $[RO(CH_2)_nOC(OH)=OH_2]^+$ (XXIV) and $[RO(CH_2)_n OCCH_2CH_2]^+$ (XXVIII) are useful. Classes of diol lipids consisting of mixtures of compounds having various chain lengths, but being derived from the same diol, can be recognized by mass spectrometry.

The present study demonstrates that neutral diol lipids, such as alk-1-enyl ether esters, alkyl ether esters and diesters of short chain diols can readily be identified and differentiated by mass spectrometry. Also quantitative estimates are possible. In conjuction with chromatographic methods, the use of mass spectrometry for the analysis of complex natural mixtures of diol lipids appears feasible.

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Identification and Analysis of Wax Esters by Mass Spectrometry[1]

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ABSTRACT

Several ions in the mass spectra of wax esters were related to the molecular structures. Assigned structures of ions were confirmed by deuterium labeling. A simple, direct method for quantitative analyses of mixtures was developed. The method involved a comparison of sets of three ions, RCO_2H^+, $RCO_2H_2^+$ and $[R'\text{-}1]^+$ from all compounds in the mixture. The method was found applicable for mixtures of unsaturated wax esters after reduction with tetradeuterio hydrazine.

INTRODUCTION

The mass spectra of esters of long chain fatty acids and short chain alcohols (1) and of short chain acids and long chain alcohols (2) have been thoroughly studied and reviewed (3,4). Although they occur widely in nature, esters of long chain acids and alcohols have received much less attention and the mass spectrum of only one such wax ester has been published (3) previously. Wax esters are encountered in the lipids of a wide variety of species and they occur in a range from C_{26} to C_{40} and in a variety of isomers. The mass spectra of a series of synthetic wax esters were measured and studied for the purposes of identification and of quantification of individual components in mixtures of such esters. The spectra of some specifically labeled wax esters were recorded to determine the atomic composition of some ions.

Shorthand formulae for wax esters have been used, i.e., hexadecyl octadecanoate is denoted as 16-18, listing the alcohol moiety first. However, in the general formula RCO_2R', R and R' are the alkyl moieties of the acyl group and the alcohol group, respectively.

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EXPERIMENTAL PROCEDURES

The mass spectra were obtained on a Hitachi Perkin-Elmer RMU-6D instrument at 70 eV and a pressure of approximately 10^{-7} torr. Wax esters, individually or in mixtures, were introduced through the direct inlet system. The spectra were recorded at temperatures that gave constant total ion concentration between 60 C and 250 C, depending on molecular weight. High resolution mass spectra were measured on an AEI MS9 double focussing mass spectrometer.

Mixtures of wax esters were analyzed by temperature-programmed gas chromatography mass spectrometry, using a 0.3 x 90 cm column packed with Gas Chrom P (100-120 mesh) coated with 2% Versamid 900.

Long chain acyl chlorides and alcohols were purchased from The Hormel Institute Lipids Preparation Laboratory. Deuterium-labeled fatty acids and 2,2-dideuteriotetradecanol were prepared as previously described (5). 1,1-Dideuterio-octadecanol was prepared by reduction of methyl octadecanoate with lithium aluminum deuteride (6). Wax esters labeled in the acyl moiety were synthesized by heating a mixture of the labeled fatty acid and an excess of the long chain alcohol at about 1 mm and 80-90 C for 3-4 hr in the presence of *p*-toluenesulfonic acid as catalyst. Other wax esters, nonlabeled or labeled in the alcohol moiety, were synthesized as previously described (7), or were prepared by treating the alcohol with excess acyl chloride for 3-4 hr at 1 mm and 60 C. The esters were purified by preparative thin layer chromatography (TLC) and recrystallized twice from petroleum ether. Yields and melting points are given in Table I.

A synthetic mixture of isomeric unsaturated wax esters, octadecyl octadec-9-enoate, 56.5 mg, and octadec-9-enyl octadecanoate, 64.1 mg, was reduced with tetradeuterio hydrazine as previously described (8,9). Residual unreacted wax esters amounted to about 20%, judging from a peak at m/e 534, and were removed by subjecting a 20 mg portion to ozonolysis (10). The mass spectrum of the product (10.1 mg) which was obtained after preparative TLC, had no peak at the molecular weight of the parent unsaturated esters (m/e 534) but a prominent molecular ion for the

TABLE I

Melting Points and Yields of Wax Esters[a]

Ester	Alcohol-acid	Melting points, C	Yield
Octadecyl 2,2-dideuteriotetradecanoate	18-14(2,2-d_2)	51.8	84%
Octadecyl 3,3-dideuteriopentadecanoate	18-15(3,3-d_2)	55.2-55.6	90%
Octadecyl 4,4-dideuteriohexadecanoate	18-16(4,4-d_2)	59.5-60	62%
1,1-Dideuteriooctadecyl octadecanoate	18(1,1-d_2)-18	52.5-52.9	95%
2,2-Dideuteriotetradecyl octadecanoate	14(2,2-d_2)-18	61.5-61.8	60%
Octadecyl dodecanoate	18-12	43.5-44	59%
Octadecyl decanoate	18-10	37.5	43%
Dodecyl octadecanoate	12-18	44.4-44.8	74%
Decyl octadecanoate	10-18	37.5-38	74%
Octyl octadecanoate	8-18	32	66%
Hexyl octadecanoate	6-18	27.5-28	61%
Butyl octadecanoate	4-18	27	59%
Propyl octadecanoate	3-18	30.5-31	33%

[a]See also reference 7.

dideuterio- addition product (m/e 536).

RESULTS AND DISCUSSION

Stenhagen et al. (3) found the diagnostically important ions M^+, $RCO_2H_2^+$, $CO_2R'^+$ and $[R' - 1]^+$. Our deuterium-labeled and non-labeled wax esters produced ions agreeing with the previously postulated (3) structures. In addition, five more characteristic ions were observed and their structures have been postulated. The elemental compositions of these ions were confirmed by high resolution measurements in the case of octadecyl octadecanoate as follows: ion $[RCO_2H\text{-propyl}]^+$, measured 241.21625, calculated 241.21674; $[R'-1]^+$, 252.28096, 252.28168; RCO^+, 267.26710, 267.26880; RCO_2H^+, 284.27212, 284.27173; $RCO_2H_2^+$, 285.27845, 285.27954; $CO_2R'^+$, 297.27872, 297.27954; $CH_2{=}COH\text{-}OR'^+$, 313.30890, 313.31055.

$RCO_2H_2^+$

This ion was found to be the base peak in all

TABLE II

Intensities of Characteristic Ions Containing the Alcohol Moiety in Mass Spectra of Saturated Wax Esters, RCO_2R', Expressed as Per Cent of $RCO_2H_2^+$ to Indicate Dependence of Abundance Upon Chain Length

Wax ester		$[R'-1]^+$		$CO_2R'^+$		$CH_2{=}C(OH)^+\text{-}O\text{-}R'$		$CH_2{=}C(OH_2)^+\text{-}O\text{-}R'$	
Alcohol	Acid	Mass	Intensity	Mass	Intensity	Mass	Intensity	Mass	Intensity
18	14(2,2-d_2)	252	21.9	297	5.0	314	1.3	315	1.2
18	15(3,3-d_2)	252	25.4	297	6.0	312	1.2	313	1.5
18	16(4,4-d_2)	252	24.6	297	6.2	313	1.2	314	2.2
18(1,1-d_2)	18	254	29.8	299	7.1	314	1.5	315	2.7
14(2,2-d_2)	18	[a]	43.9[a]	243	7.2	258	1.9	259	5.0
18	10	252	17.7	297	4.3	312	0.8	313	0.3
18	12	252	20.5	297	5.0	312	1.0	313	0.6
18	14	252	22.7	297	4.8	312	1.1	313	1.1
18	16	252	26.3	297	6.1	312	2.3	313	1.8
18	18	252	27.7	297	6.6	312	1.4	313	2.7
16	18	224	36.6	269	6.7	284	[b]	285	[b]
14	18	196	53.0	241	12.8[c]	256	1.4	257	5.5
12	18	168	70.5	213	8.5	228	1.5	229	6.4
10	18	140	91.7	185	13.5	200	1.7	201	9.8
8	18	112	113.3	157	8.4	172	2.1	173	10.5
6	18	84	125.6	129	22.6	144	7.3	145	13.7
4	18	56	194.9	101	20.5	116	44.1	117	29.2
3	18	42	[d]	87	38.9	102	160.0	103	68.6

[a]$[R'-D]^+ + [R'-H]^+$.
[b]Coinciding with RCO_2H^+ and $RCO_2H_2^+$.
[c]Coinciding with $[RCO_2H\text{-propyl}]^+$.
[d]Not present. The base peak was found at m/e 61, 359%.

TABLE III

Intensities of Characteristic Ions Containing the Acyl Moiety in Mass Spectra of Saturated Wax Esters, RCO_2R', Expressed as Per Cent of $RCO_2H_2^+$ to Indicate Dependence of Abundances Upon Chain Length

Wax ester		$[RCO_2H\text{-propyl}]^+$		RCO^+		RCO_2H^+		$RCO_2H_2^+$[a]		M^+	
Alcohol	Acid	Mass	Intensity	Mass	Intensity	Mass	Intensity	Mass	Intensity	Mass	Intensity
18	14(2,2-d_2)	[b]	[b]	213	11.9	230	28.1	231	100	482	23.1
18	15(3,3-d_2)	199	3.6	227	10.9	244	26.6	245	100	496	21.2
18	16(4,4-d_2)	213	3.9	241	9.5	258	28.5	259	100	510	27.4
18(1,1-d_2)	18	241	4.7	267	9.4	284	22.0	285	100	538	35.3
14(2,2-d_2)	18	241	5.0	267	11.5	284	19.8	[c]	100	482	28.7
18	10	129	7.3	155	14.6	172	17.1	173	100	424	12.8
18	12	157	4.2	183	12.4	200	19.1	201	100	452	15.9
18	14	185	5.8	211	11.1	228	20.7	229	100	480	17.4
18	16	213	4.2	239	10.6	256	22.8	257	100	508	27.9
18	18	241	4.7	267	10.2	284	24.4	285	100	536	30.2
16	18	241	5.3	267	10.9	284	24.6	285	100	508	30.6
14	18	241	12.8[d]	267	12.8	284	26.1	285	100	480	24.9
12	18	241	8.8	267	15.3	284	27.5	285	100	452	30.8
10	18	241	9.0	267	19.5	284	30.1	285	100	424	38.3
8	18	241	11.4	267	25.1	284	32.7	285	100	396	34.2
6	18	241	10.9	267	31.8	284	32.2	285	100	368	36.0
4	18	241	18.5	267	57.5	284	31.2	285	100	340	61.4
3	18	241	29.2	267	111.9	284	29.2	285	100	326	127.7

[a]Base peak in all spectra except in those of 3, 4, 6, 8-18.
[b]m/e 185, 186, 187: 1.37, 1.81, 1.72.
[c]$RCO_2H_2^+ + RCO_2HD^+$.
[d]Coinciding with $CO_2R'^+$.

cases except those in which the alcohol moiety was shorter than 10 carbon atoms (Tables II and III). It is apparently formed directly from the parent ion, M^+, judging from a corresponding metastable peak. Hence, the cleavage of the O-R′ bond and the transfer of two hydrogens from the R′ moiety must be a concerted reaction. Using deuterium-labeled *n*-butyl propionates, Djerassi et al (11) established that in these compounds the double hydrogen transfer involved positions 2 and 3 of the alkoxy group to a higher degree than any other position. The spectra of wax esters labeled in positions 1 or 2 of the alcohol moiety revealed increased $(RCO_2H+1)^+$ ions indicating transfer of some deuterium. The ratio $RCO_2H_2^+$: RCO_2HD^+ was found to be 90:10 and 77:23, respectively.

RCO_2H^+

The ion formally equivalent to the carboxylic acid might be formed by a McLafferty rearrangement. Djerassi et al. (11) found, however, that in the cases of butyl propionate and benzoate the transferred hydrogen originated to some extent from positions other than the 2 position of the alkyloxy group. The spectrum of 2,2-dideuteriotetradecyl octadecanoate showed the ratio of RCO_2H^+:RCO_2D^+ to be 85:15. This implies that alkoxy fission with transfer of one hydrogen atom from position 2 (McLafferty rearrangement) is responsible for this ion only to a small degree.

$[R'-1]^+$

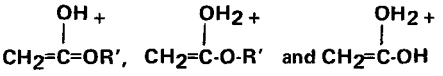
The ratio of the ions $[R'-H]^+$ to $[R'-D]^+$ in the spectrum of 2,2-dideuteriotetradecyl octadecanoate was found to be 70:30, indicating that the main portion of the ion $[R'-1]^+$, is not formed in a McLafferty rearrangement with charge retention on the hydrocarbon. This corresponds with the finding of Djerassi et al. (11) for butyl benzoate that only 25% of this ion is produced by a McLafferty rearrangement. At least part of this ion is produced by fragmentation of $[CH_2{=}COH\text{-}OR']^+$ since a corresponding metastable peak was always found.

RCO^+ and $CO_2R'^+$

These ions are formed by normal cleavage (12) of the bonds adjacent to the carbonyl group. Absence of $[RCO\text{-}1]^+$ and $[CO_2R'\text{-}1]^+$ ions in the spectra of deuterium-labeled wax esters indicates that if scrambling takes place between the alkyl moieties, RCO^+ and $CO_2R'^+$ are formed prior to the scrambling. This agrees with reports for diglycerides (13) and triglycerides (5) labeled in the acyl moieties in which the deuteriums apparently were also confined to their original acyl groups.

$[RCO_2H\text{-}C_3H_7]^+$

All unlabeled wax esters exhibited a relatively weak ion 43 mass units lower than the RCO_2H^+ ions. In the cases where positions 3 and 4 of the acyl group were *gem.* deuterated this interval increased to 45 mass units. When the 2 positions were occupied by deuteriums a cluster corresponding to loss of 43, 44 and 45 was observed. This pattern is analogous to earlier findings for long chain methyl esters (14,15) and triglycerides (5) which apparently expel three methylene groups adjacent to the carbonyl group plus one hydrogen. A similar extrusion must take place with wax esters too. The cluster of three peaks in the case of labeled 2 positions is the result of partial loss of deuterium due to exchange reactions. Deuterium adjacent to the carbonyl group in long chain methyl esters (14,15) and triglycerides (5) is known to be exchangeable with hydrogen atoms in positions 5, 6 and 7 of the acyl chain. In the case of 14-18 this ion coincided with $CO_2R'^+$ at m/e 241, relative intensity 12.8%. These ions were found at different masses, m/e 241 and 243, respectively, in the spectrum of 14(2,2-d_2)-18, and the sum of their relative intensities, 12.2%, is nearly the same as that of the double ion.



The formation of the first two ions involves

TABLE IV

Quantitative Analyses of Mixtures of Wax Esters

Alcohol-acid	Molecular weight	Gravimetric composition	Found %
18-16	508	48.3	48.9
18-16(4,4-d_2)	510	51.7	51.1
18-10	424	49.4	52.1
10-18	424	50.6	47.9
18-12	452	41.1	43.4
12-18	452	58.9	56.6
18-14	480	46.4	47.9
14-18	480	53.6	52.2
18-16	508	62.3	62.8
16-18	508	37.7	37.2
14-16	452	34.3	34.7
18-12	452	27.0	27.2
12-18	452	38.7	38.1
16-14	452	21.0	22.0
14-16	452	27.1	26.4
18-12	452	21.3	20.9
12-18	452	30.6	30.8

transfer of one and two hydrogen atoms, respectively, from the acyl moiety. Both ions showed retention of the deuterium atoms when the position adjacent to the carbonyl group was doubly labeled as in 18-14(2,2-d_2), suggesting that fission of the β-bond occurred prior to exchange reactions. Retention of these labile deuterium atoms indicates that a McLafferty rearrangement is involved (5,14,15). Further evidence for such a rearrangement was found in the retention of one deuterium atom in both ions when positions 4 of the acyl moiety were labeled. The origin of the second hydrogen transferred is unknown.

The base peak in the spectrum of propyl stearate was found at m/e 61 rather than the expected m/e 42 ($[R'-1]^+$, see Table II). This ion,, $[CH_2=C(OH_2)-OH]^+$, which involves a triple hydrogen transfer (16,17), is probably formed from $[CH_2=C(OH)-O-R']^+$, m/e 102, since a corresponding strong metastable peak was found at m/e 36.5 (Calculated 36.48). This ion was also found in spectra of other wax esters but to a much smaller extent. The increased relative intensity of this ion in the case of propyl might be due to absence of steric hindrance.

Quantitative Analysis of Mixtures

Since several of the pronounced, characteristic ions of wax esters are readily recognized, a method was sought for quantitative analyses of mixtures. Analyses of mixtures by means of mass spectrometry have been performed primarily on hydrocarbons (18,19) and amino acids (20), but this method should be applicable to lipids as well.

Wax Esters Having the Same Molecular Weight. Ions characteristic of each wax ester component in model mixtures were used singly or in combination as bases of calculations of composition. The most favorable results were obtained when the sums of the heights of the ions RCO_2H^+, $RCO_2H_2^+$ and $[R'-1]^+$ for each component were compared. The following relationship was derived and empirically verified:

$$\%a = \frac{100\ \Sigma_a}{\Sigma_a + \Sigma_b + \ldots \Sigma_n}$$

where Σ_a = the sum of the peak heights of ions RCO_2H^+, $RCO_2H_2^+$ and $[R' - 1]^+$ for component a, etc. The results obtained using this method are given in Table IV. Calculations based upon individual ions gave greater deviations from true values. For instance, the observed content of 18-10 (49.4%) in the mixture (18-10, 10-18) was calculated to be 22.9%, 65.6% and 52.3% when ions $[R'-1]^+$, $RCO_2H_2^+$ and RCO_2H^+, respectively, were used as the single bases of calculation, whereas 52.1% was calculated (Table IV) using the formula. Differences in running conditions did not significantly influence the results either. The mixture (18-16, 16-18) was repeated one month later and the composition, 62.1% and 37.9%, was found to be consistent with the earlier finding, 62.8% and 37.2% (Table IV). Several more examples are given in Table IV proving the validity of the method. It must, however, be used with caution when one or several of the components of the mixture are present in very small quantities.

Wax esters Having Different Molecular Weights. The method outlined above was not applicable for wax esters having different numbers of carbon atoms. For instance, the four-component mixture (18-18, 18-16, 18-14, 18-12) gave 6.9%, 11.5%, 31.0% and 60.5%, respectively, whereas the gravimetric composition was 22.9%, 19.5%, 29.9% and 28.4%. The deviations are probably due to differences in volatility. Wax esters having different molecular weights are, however, readily separated by chromatographic (GLC) methods (21). Combined gas chromatography-mass spectrometry was applied on a synthetic mixture (18-18, 18-14, 14-18) with known composition. Using the method outlined above, the mass spectrum obtained for the GLC peak representing 18-14 and 14-18 gave the ratio 46.1:53.9 whereas the known composition was 43.9:56.1.

Unsaturated Wax Esters. Unsaturated wax esters can be separated by argentation chromatography into groups having different numbers of double bonds (22). Subsequent deuteration of the double bonds with tetradeuterio hydrazine followed by gas chromatography-mass spectrometry allows quantitative analysis of each group provided residual unreduced esters have been removed either by argentation chromatography (22) or by ozonolysis (10).

A synthetic mixture consisting of 18-18:1 and 18:1-18 was treated accordingly. When the contribution of ^{13}C and the presence of an $[M-1]^+$ ion (2.2% of M^+) in the spectrum of 18-18 were taken into account, the ratio of d_0, d_1 and d_2 in the wax esters were 3.3:17.8:78.8. The peak heights of the appropriate ions were corrected for isotopic impurities (^{13}C, d_0 and d_1 wax esters) giving m/e 252 29.1 mm, m/e 254 22 mm, m/e 284 27 mm, m/e 285 113.2 mm, m/e 286 23 mm and m/e 287 91mm. Using the formula described above, the content of 18 d_2-18 was thus found to be 53.1% [100(22 + 27 + 113.2/305.3)]. The original mixture consisted of 18-18:1 and 18:1-18 in

the ratio 46.8:53.2. Since all the ions used in the calculation are formed by loss or capture of one or two hydrogens, an isotope effect might interfere when certain double bonds in the chain are deuterated.

ACKNOWLEDGMENTS

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Metabolism of Ceramide Phosphorylethanolamine, Phosphatidylinositol, Phosphatidylserine and Phosphatidylglycerol by Housefly Larvae[1]

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ABSTRACT

Microsome preparations (40,000-90,000 g sediment) from *Musca domestica*, housefly, larvae convert exogenous ^{32}P-labeled phosphatidylinositol, phosphatidylserine and phosphatidylglycerol to the respective lysoglycerophosphatides and, ultimately, to the glycerophosphoryl derivatives. These data, combined with previous results, demonstrate that housefly larvae can convert their normal diacylglycerophosphatides to the respective glycerophosphoryl derivatives. Experiments utilizing exogenous ^{3}H-labeled, ^{32}P-labeled and ^{14}C-labeled ceramide phosphorylethanolamine demonstrate that particulate preparations from housefly larvae convert ceramide phosphorylethanolamine to ceramide, phosphorylethanolamine, sphingosine and fatty acid. The presence of ceramide phosphorylethanolamine phosphohydrolase and ceramidase activity in housefly larvae is consistent with the conclusion that ceramide phosphorylethanolamine is metabolized to ceramide and phosphorylethanolamine and the ceramide is then hydrolyzed to sphingosine and fatty acid. Thus, metabolism of ceramide phosphorylethanolamine by these insects is analogous to the metabolism of sphingomyelin by mammalian systems.

INTRODUCTION

Phosphatidylcholine and phosphatidylethanolamine are the principal phospholipids of many insects (1). Housefly (2) and blowfly (3) larvae also contain minor phospholipids such as phosphatidylserine and phosphatidylinositol as well as ceramide phosphorylethanolamine-type sphingolipids (2,4-6). Particulate preparations, containing microsomes, from larvae of the housefly (*Musca domestica*) (7,8), the blowfly (*Phormia regina*) (7) and the mosquito (*Papiens fatigans*) (9,10), contain phospholipase A^1 and A^2 activity towards phosphatidylcholine and phosphatidylethanolamine. Little is known concerning the metabolism of the minor lipids of insects. We will demonstrate that particulate preparations containing microsomes from housefly larvae deacylate phosphatidylserine, phosphatidylinositol and phosphatidylglycerol to the respective lysophosphatides and, ultimately, to the water soluble glycerophosphoryl derivatives. Ceramide phosphorylethanolamine is cleaved to ceramide and phosphorylethanolamine, and the ceramide is cleaved to fatty acids and sphingosines.

METHODS

Isolation of Phospholipids and Ceramide Derivatives

^{32}P-Labeled phosphatidylinositol, phosphatidylglycerol, phosphatidylserine and ceramide phosphorylethanolamine were isolated from housefly larvae that had been reared on a ^{32}Pi-containing diet as described previously (6,8). The lipid extract containing phosphatidylinositol, phosphatidylserine, phosphatidylglycerol and other phospholipids was chromatographed on silicic acid to remove neutral lipids and lecithin as described elsewhere (8,11). The remaining phospholipid classes were resolved by column chromatography using DEAE cellulose essentially as described by Rouser et al. (12). Column eluates containing acetic acid or formic acid were dripped directly into NH_4HCO_3 solutions to neutralize the acid. These preparations were then partitioned between water and chloroform and the chloroform phase evaporated to dryness. The dried lipid samples were dissolved in warm tertiary butyl alcohol, lyophilized and stored dry at -20 C. After chromatography on DEAE cellulose, phosphatidylglycerol and phosphatidylinositol were separated by preparative thin layer chromatography (TLC) using Brinkman preparative plates (Silica Gel F-254) and the solvent system, chloroform-methanol-H_2O (65:35:4). Occasionally, the other phospholipids were further purified by preparative TLC using the solvent system, chloroform-methanol-H_2O (65:35:4) or chloroform-methanol-conc NH_4OH (65:35:4). The phospholipids were at least 95% pure before use, as determined by monitoring the ^{32}P distribution

[1]Paper No. 5250 from the Michigan Experiment Station.

with a Packard Model 7201 radiochromatogram scanner after TLC in one or more solvent systems. In experiments (unpublished data), G.R. Hildenbrandt has shown that the phosphorus-inositol ratio and the phosphorus-serine ratio of the glycerophosphoryl derivatives prepared from these preparations of phosphatidylinositol and phosphatidylserine are 1.0 ± 0.1. ^{32}P in column eluates was monitored with a recording Geiger Muller apparatus.

Tritiated ceramide phosphorylethanolamine was prepared according to the procedure described above for preparation of ^{32}P-labeled lipids except that larvae were reared on a diet containing 1 curie of tritiated water. ^{14}C-labeled ceramide phosphorylethanolamine was prepared similarly except that larvae were reared on diets containing uniformly labeled L-serine from New England Nuclear. Ceramide was prepared from bovine heart sphingomyelin by treatment with 2-5 mg of phospholipase C from Worthington, *Clostridium Welchii*, as follows. The sphingomyelin (400 mg) in chloroform was adsorbed onto 1 g of Celite using a rotary evaporator and the reaction was run overnight with stirring at room temperature in 20 ml of 0.025 *M* Hepes (N-2-hydroxypiperazine-N'-2-ethane sulfuric acid), pH 7.3, containing 3 mM $CaCl_2$. The chloroform soluble-products were resolved on silicic acid (8). Ceramide was detected on thin layer chromatograms utilizing a Chloroxbenzidine spray reagent (13). Sphingosine standards were prepared by hydrolyzing bovine brain sphingomyelin in 1 N methanolic HCl for 18 hr at 105 C. Phospholipids were deacylated by adding 1.0 ml of 0.5 N KOH in 95% methanol to 2 ml of phospholipid in chloroform. After stirring for 10 min at room temperature, excess Dowex-50 resin, H^+ form, was added and the samples were partitioned between equal volumes of chloroform and water. The resin was removed and the aqueous phase neutralized immediately with NaOH. Glycerophosphate and Pi were removed by column chromatography on Dowex-1, formate form and the water soluble glycerophosphoryl derivatives were then chromatographed on Dowex-50 H^+ as described below.

Enzyme Preparations and Assays

Microsomes were prepared from housefly larvae and phospholipase assayed as described by Kumar et al. (8). ^{32}P-labeled diacylglycerophosphatides were separated from the respective monoacylglycerophosphatides by TLC as described in the figure legends. The water soluble portion of the reaction products, which contained the glycerophosphoryl derivatives,

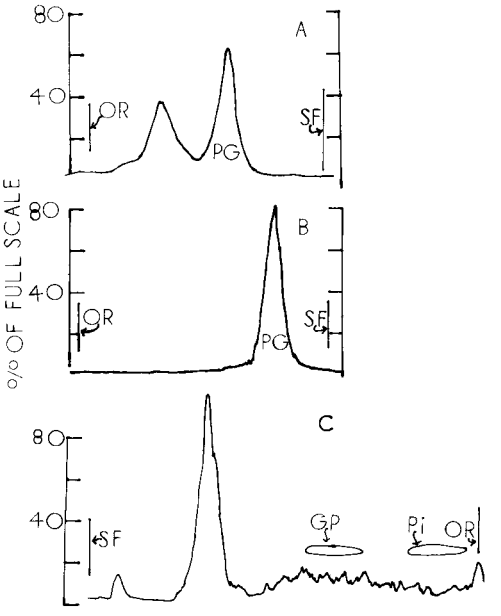

FIG. 1. Tracings of the radiochromatogram scans of chromatograms of the chloroform soluble and water soluble extracts obtained from larval microsomes incubated with ^{32}P-labeled phosphatidylglycerol. Larval microsomes, 0.6 mg protein, were incubated with 1 μmole of ^{32}P-labeled phosphatidylglycerol (44,000 CPM) for 1 hr at 31 C in 1 ml of 50 mM Tris HCl, pH 7.2. Assays were performed as described in Methods. A contained 2.5 mM lauryl sulfate. Lauryl sulfate and microsomes were not added in B. A and B are radiochromatogram scans of the chloroform phases that were chromatographed on Silica Gel F-254 Brinkman thin layer plates in the solvent system, chloroform-methanol-H_2O (65:35:4). 73% of total counts were in the chloroform phase of A and 100% of total counts were in the chloroform phase of B. C is a radiochromatogram scan of the water soluble fraction from A (27% of total counts) that were chromatographed on paper in the solvent system, methanol-conc. NH_4OH-H_2O (12:2:3). OR, origin; SF, solvent front; PG, phosphatidylglycerol; GP, α-glycerophosphate; Pi, inorganic phosphate.

was passed through columns of Dowex-50 resin, H^+ form, 100-200 mesh, 0.8 x 10 cm to remove cations. The column eluates (containing the glycerophosphorylinositol, -serine, or -glycerol) were lyophilized, dissolved in a small quantity of water (<0.5 ml), neutralized, and then chromatographed on paper as described in the legends of Figures 1-3. ^{32}P in water, in organic solvents and on silicic acid was quantitated utilizing Cerenkov radiation as described by Haviland and Bieber (14). Ceramide phosphorylethanolamine phosphohydrolase was prepared and measured as described in the legend of Figure 5.

^{32}Pi was determined by the method of Lindberg and Ernster (15). Pi was precipitated from

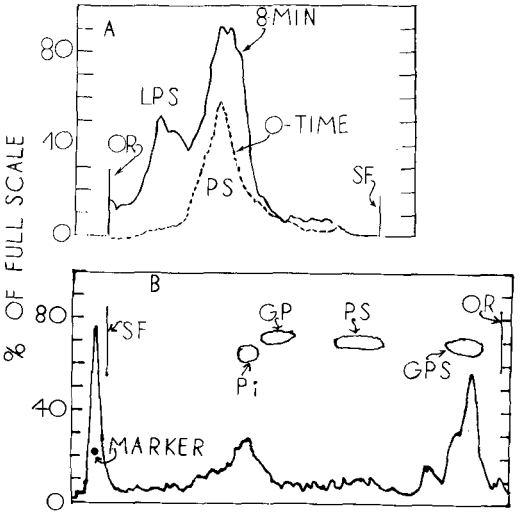

FIG. 2. Tracings of the radiochromatogram scans of chromatograms of the chloroform soluble and water soluble extracts obtained from larval microsomes incubated with ^{32}P-labeled phosphatidylserine. Microsomes, 2.9 mg protein, were incubated for 8 min in 2.5 ml of a solution containing 0.05 M imidazole buffer, pH 7.4, 2.5 mM lauryl sulfate, 1 mM $HgCl_2$, and 2.75 μmoles of phosphatidylserine (64,400 CPM). The reaction was terminated and assayed as described in the Methods. The aqueous phase contained 7,762 CPM. SF, solvent front; OR, origin. For A, the chloroform soluble extract was chromatographed as described for Figures 1A and B. LPS, lysophosphatidylserine; PS, phosphatidylserine. For B, the water soluble fraction was chromatographed on paper in the solvent system, picric acid-tertiary butyl alcohol-water (4 g:80:20). GP, α-glycerophosphate; GPS, glycerophosphorylserine; PS, phosphorylserine; Pi, inorganic phosphate.

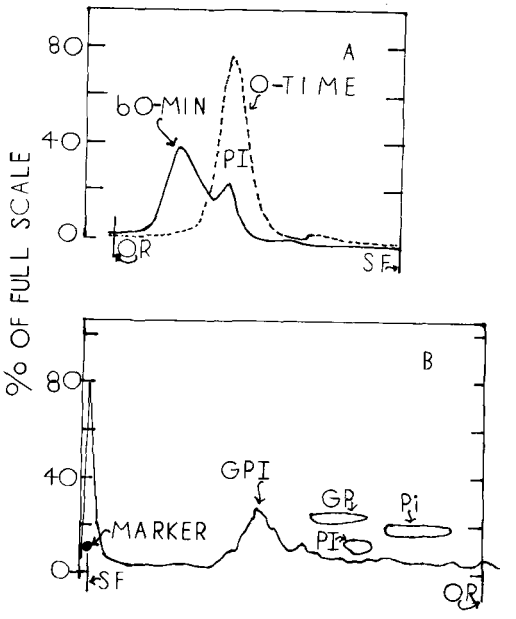

FIG. 3. Tracings of radiochromatogram scans of chromatograms of the chloroform soluble and water soluble extracts obtained from larval microsomes incubated with ^{32}P-labeled phosphatidylinositol. Incubations and assays were identical to those described for Figure 1 including quantity and specific radioactivity of substrate. For A, the lipid extract was chromatographed as in Figures 1A and B. The dashed curve (– – –) is a tracing of the radiochromatogram scan obtained with the lipid extract (100% of total counts) from an incubation without microsomes, and the solid curve (——) is a tracing of the radiochromatogram scan of the lipid extract (66% of total counts) from microsomes that were incubated 60 min with 2.5 mM lauryl sulfate. PI, phosphatidylinositol; 0.7 mg protein per sample was used. For B, an aliquot of the water soluble portion (53% of total counts) of a 90 min incubation, as described for Part A, was chromatographed on paper in the solvent system described for Figure 1C. B is a tracing of the radiochromatogram scan. GPI, glycerophosphorylinositol; GP, α-glycerophosphate; PI, phosphorylinositol; and Pi, inorganic phosphate. In A and B, SF, solvent front; OR, origin.

aqueous solutions as the triethylamine phosphomolybdate complex by the method of Sugino and Miyoshi (16). Serine was quantitated using an automated amino acid analyzer as described by D.C. Robertson, H.B. Brockman, W.I. Wood and W.A. Wood (personal communication). Protein was determined by the method of Lowry et al. (17).

Inositol was quantitated by gas chromatography of the TMSi derivatives as described by Wells et al. (18).

RESULTS

Microsomal Deacylation of Phosphatidylinositol, Phosphatidylserine, and Phosphatidylglycerol

When microsomes from housefly larvae were incubated with ^{32}P-labeled phosphatidylserine and phosphatidylglycerol, TLC of the chloroform soluble fraction demonstrated the presence of the respective ^{32}P-labeled lysophospholipids and paper chromatography of the water soluble fractions demonstrated production of glycerophosphoryl derivatives (Fig 1 and 2). The thin layer chromatograms for Figures 1A and B were run in the solvent at different times. Thus, the difference in the Rf of phosphatidylglycerol in Fig. 1A and B is probably due to slightly different solvent composition or different activation of the plates. Some inorganic phosphate was present in the water soluble fraction when phosphatidylserine was the substrate (Fig. 2B). The inorganic phosphate was probably produced by the phosphatase(s) which are present in the enzyme preparations. Glycerophosphate, a phosphatase substrate, could be produced enzymatically

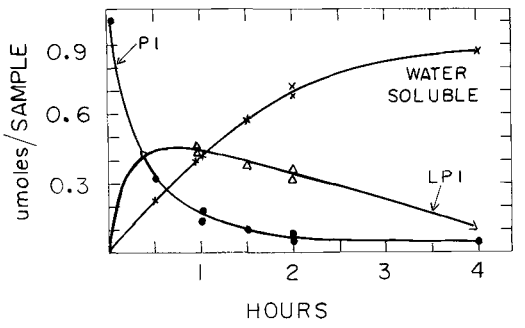

FIG. 4. Time course for deacylation of ^{32}P-labeled phosphatidylinositol by housefly larvae microsomes. Each sample of 1 ml, taken from an 8 ml incubation mixture at the indicated times, contained: 1 μmole ^{32}P-labeled phosphatidylinositol at zero time; 50 μmoles Tris Cl$^-$, pH 8.0 and 2.5 μmoles lauryl sulfate. Incubation was at 31 C, and assays were performed as described in Methods. PI, phosphatidylinositol, ●—●; LPI, lysophosphatidylinositol, △—△; water soluble, ^{32}P-labeled glycerophosphorylinositol formed, x—x. The protein concentration was 0.7 mg/ml.

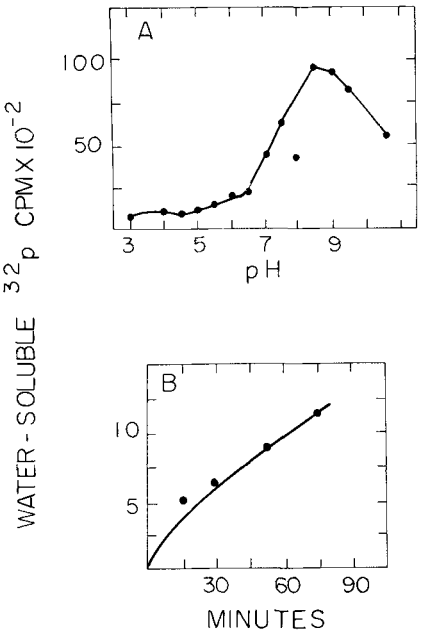

FIG. 5. pH Optimum and Time course for hydrolysis of ^{32}P-labeled ceramide phosphoryl-ethanolamine. In A, 0.2 ml of the 10,000-40,000 g fraction (0.6 mg protein) in 0.1 M KCl was added to 0.8 ml of 0.05 M buffer, and 0.1 ml of ^{32}P-labeled ceramide phosphorylethanolamine in 5% Triton X-100. The final concentration of Triton X-100 was 1% and ceramide phosphorylethanolamine was 0.25 mM = 32,140 CPM of ^{32}P. The samples were incubated at 31 C for 30 min, and the reaction was terminated by adding 6 ml of chloroform-methanol (1:2). After mixing for 15 min, 5 ml of chloroform and 5 ml of 0.02 M $MgCl_2$ were added. The samples were mixed and the layers separated by centrifugation. The water soluble fraction was counted and used as a measure of the extent of reaction. The water soluble CPM are plotted in A and B. The buffers were formate at pH 3.1 and 4.1· acetate at pH 4.5 and 5.5; citrate at pH 5.0; imidazole at pH 6.0, 6.5 and 7.0; Tris Cl$^-$ at pH 7.5, 8.5 and 9.5; phosphate at pH 8.0, and glycine at pH 9.0 and 10.5. In B, 1 ml of the 10,000-40,000 g pellet in 0.05 M KCl was added to 4 ml of 0.05 M Tris HCl, pH 9.5, buffer containing sufficient Triton X-100 and ceramide phosphorylethanolamine to make a final concentration of 1% Triton X-100 and 0.3 mM ^{32}P-labeled ceramide phosphorylethanolamine. The sample was incubated at 31 C. 1.0 ml Aliquots were taken at the times indicated and assayed as described for part A. Each assay contained 0.6 mg protein. The 10,000-40,000 g fraction was prepared by homogenizing larvae in 0.15 M Tris, pH 7.5 (5ml/g larvae) in a micro Waring blendor for 30 sec at maximum speed. The homogenate was squeezed through several layers of cheesecloth and a 10,000-40,000 g fraction was collected and suspended in 0.1 M KCl or the appropriate buffer.

from glycerophosphorylserine by traces of phosphodiesterase. A large amount of this phosphodiesterase activity is present in the 90,000 g supernatant fluid from larvae and occasionally small amounts remain with the microsome preparations. Attempts to detect production of lysophosphatidylserine were unsuccessful until lauryl sulfate and $HgCl_2$ were added to the reaction mixture. It was shown previously (8) that lauryl sulfate greatly stimulates monoacylglycerophosphatide production from the diacylglycerophosphatides. Apparently, in the absence of lauryl sulfate and $HgCl_2$, housefly microsomes deacylate lysophosphatidylserine at a much greater rate than phosphatidylserine, preventing accumulation of the lyso-intermediate. When ^{32}P-labeled phosphatidylinositol was the substrate, after 1 hr, microsome preparations had converted much of the ^{32}P to a phospholipid which had the Rf on thin layer chromatograms of lysophosphatidylinositol (Fig. 3A). A water soluble compound was also formed. It had an Rf corresponding to that of glycerophosphorylinositol rather than glycerophosphate, inorganic phosphate or phosphorylinositol (Fig. 3B).

Since phosphatidylinositol is cleaved to phosphorylinositol and diglyceride in some systems, its cleavage by the insect system was further investigated. The microsomal system from housefly larvae is capable of deacylating most of the added phosphatidylinositol, as shown by the time course in Figure 4. At the end of the experiment, 4 hr, the concentration of exogenous phosphatidylinositol had decreased from 1 mM to 0.04 mM. The amount of lysophosphatidylinositol increased rapidly and then decreased with time, while the glycerophosphorylinositol only increased with time, indicating a product-precursor relationship.

Alternate pathways of phosphatidylinositol

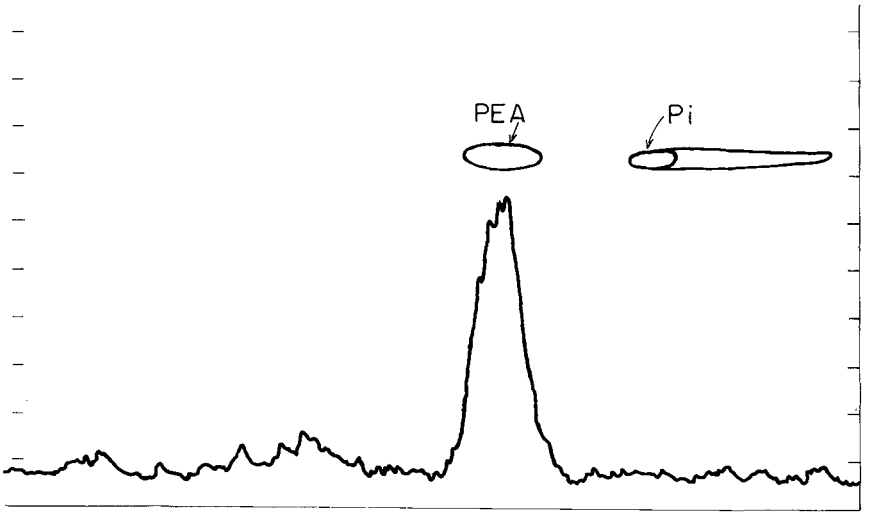

FIG. 6. Identification of ^{32}P-labeled phosphorylethanolamine as a product of the cleavage of ceramide phosphorylethanolamine. To a 10,000-40,000 g fraction, 0.6 mg protein, in 0.5 ml of 0.05 M KCl was added 0.5 ml of 0.5 M Tris Cl, pH 9.0 and 0.2 ml of ^{32}P-labeled ceramide phosphorylethanolamine (0.43 μmole) in 1% Triton X-100. The sample was incubated at 31 C for 30 min. The reaction was terminated by adding 3 ml of liquid phenol; see reference 39 for description of this method. The phenol was washed two times with 2 ml water, and the combined aqueous phases were applied to a Dowex-1, HCO_3^- column. The column was eluted with water, then 0.25 M triethylammonium bicarbonate, pH 7.5, followed by 1 M triethylammonium bicarbonate, pH 7.5. Two peaks were obtained as described in the text. The first radioactive peak was chromatographed on paper using the solvent system, methanol-conc NH_4OH-H_2O (12:2:3). Figure 6 is a tracing of a portion of a radiochromatogram scan of the paper chromatogram. One radioactive peak was detected; it had an Rf identical to phosphorylethanolamine. PEA, phosphorylethanolamine; Pi, inorganic phosphate.

metabolism were not detected in housefly larvae. Attempts to detect production of phosphorylinositol using whole homogenates as well as particulate fractions containing microsomes plus lysosomes and mitochondria were unsuccessful. Assays were performed at pH 5.0 and 8.0. Such results indicate that cleavage of phosphatidylinositol to diglyceride and phosphorylinositol is not a major pathway of metabolism in housefly larvae. It should be noted that deacylation of phosphatidylinositol was detected at pH 5.0 with particulate preparations that should contain lysosomes. The water soluble fraction from the pH 5.0 incubation contained inorganic ^{32}P and glycerophosphorylinositol-^{32}P. The precursor of ^{32}Pi was not investigated and could have been phosphorylinositol.

In these experiments, the ^{32}P-labeled water soluble products were not rigorously characterized; however, paper and column chromatography demonstrated that the principal water soluble ^{32}P was not inorganic phosphate, glycerophosphate, phosphorylinositol or phosphorylserine. The water soluble ^{32}P had paper chromatographic properties expected for the glycerophosphoryl derivatives of inositol, serine and glycerol. Authentic glycerophosphoryl derivatives of inositol, serine and glycerol were prepared from the respective diacylglycerophosphatides by mild alkaline hydrolysis. Portions of these derivatives were used as standards for paper chromatography and as substrates for the phosphodiesterase that cleaves these compounds to glycerophosphate and free hydroxyl compound.

Cleavage of Ceramide Phosphorylethanolamine by Particulate Preparations Obtained From Housefly Larvae

When whole homogenates as well as the 0-800 g, 800-9,000 g, 9,000-40,000 g, 40,000-90,000 g and the 90,000 g soluble fractions from housefly larvae were incubated with ^{32}P-labeled ceramide phosphorylethanolamine at pH 7.5 and 9.5, water soluble ^{32}P was released. These experiments demonstrated that these fractions cleaved ceramide phosphorylethanolamine. The soluble fraction and the 0-800 g fraction contained the lowest specific activities. The ratio of the specific activities of the 800-9,000 g, 9,000-40,000 g and 40,000-90,000 g fractions varied from preparation to preparation. The specific activity differences appeared to be related to the stage of

TABLE I

Fraction	Total CPM	Distribution of $CHCl_3$-soluble material, %
Free fatty acid	13,320	10.2
Ceramide	2,500	1.9
Sphingosines	18,600	14.3
Ceramide phosphorylethanolamine	95,520	73.5

[a]Amounts of 0.5 mg of ^3H-ceramide phosphorylethanolamine plus 4 mg of ceramide were dissolved in 1 ml of a solution of 1% Triton X-100 and 0.05 M glycine, pH 9.0. The 8,000-40,000 g particulate fraction in 2 ml of 0.05 M glycine buffer, pH 9.5, from 21 g of larvae was added to the substrate solution and incubated at 31 C for 15 min. The reaction was terminated by adding 8 ml of chloroform-methanol, 1:2, and stirring for 15 min. Then 5 ml of 0.02 M $MgCl_2$ and 5 ml of chloroform were added. The chloroform layer was washed three times with 5 ml of 0.02 M $MgCl_2$. The aqueous washes were combined and counted. The aqueous layer contained 8,430 CPM. The chloroform fraction was evaporated to dryness, and the fatty acids, sphingosine, ceramide and ceramide phosphorylethanolamine separated by TLC as follows. The chloroform soluble material was streaked onto a Silica Gel F-254 (Brinkman) plate and developed in chloroform-glacial acetic acid (90:10). The plate was dried at approximately 50 C and placed in iodine vapors. The zones with Rf's of ceramide and oleic acid were marked. The plate was then placed into the solvent system, chloroform-methanol conc NH_4OH (65:35:4) and developed until the solvent front reached the ceramide zone. This solvent system separated ceramide phosphorylethanolamine from the sphingosines. The zones having Rf's of sphingosines, fatty acids and ceramide were scraped from the plates. The scrapings were put into small columns and eluted with methanol. Aliquots of the methanol solution were transferred to scintillation vials and the samples counted for ^3H. The remainder of the methanol was evaporated to dryness and the residue was dissolved in chloroform and applied to thin layer plates. The plates were developed in the solvent systems A, chloroformglacial acetic acid (96:4), and B, chloroform-methanol-conc, NH_4OH (65:35:4). For each, the zones with Rf's of sphingosine, fatty acid and ceramide were scraped from plates, eluted and counted as described above. The rechromatography was essential because the ceramide and sphingosine overlapped slightly on the initial chromatography. In solvent A, the Rf's of ceramide phosphorylethanolamine, sphingosine, ceramide and free fatty acids were 0.0, $<$ 0.02, 0.26, and 0.59, respectively. In solvent B, the Rf's of ceramide phosphorylethanolamine and sphingosine were 0.42 and 0.88, respectively. The tritiated samples were dissolved in 1 ml of methanol or 1 ml of water. Then 0.4 ml of Triton X-100 and 10 ml of toluene scintillation solution (4 g PPO and 100 mg POPOP per liter toluene) were added and the samples were counted. 13.9×10^4 DPM were used in the experiment.

larval development and were not investigated further. Since all three particulate fractions contained ceramide phosphorylethanolamine phosphohydrolase activity, a 10,000-40,000 g particulate fraction was used for most of the following investigations. In all of the preparations, ^{32}P-labeled phosphorylethanolamine as well as ^{32}Pi were detected when ^{32}P-labeled ceramide phosphorylethanolamine was the substrate.

pH Optimum and Time Course

Four pH optimum curves were run on 10,000-40,000 g preparations. In all four determinations, the greatest activity was obtained near pH 9.0; however, in two of the runs, considerably more activity at pH 4-5 and at 7-8 was obtained than is shown in Figure 5A.

At pH 9.5, hydrolysis of ceramide phosphorylethanolamine was nearly linear for over an hour, as shown in Figure 5B.

Identification of Phosphorylethanolamine as a Reaction Product

When ^{32}P-labeled ceramide phosphorylethanolamine was incubated with the 0-800 g, 800-9,000 g, 9,000-40,000 g, 40,000-90,000 g, or the 90,000 g supernatant fraction, water soluble ^{32}P was released. From 10% to 45% of the water soluble radioactivity from each fraction was inorganic phosphate as determined by partitioning the phosphomolybdate complex between sulfuric acid and isotubanol-benzene (15). When the water soluble fraction from a 10,000-40,000 g fraction was exchanged onto Dowex-1 in the bicarbonate form and the column eluted successively with 0.25 M and 1 M triethylammonium bicarbonate, pH 7.5, two radioactive peaks wer obtained. The first peak had chromatographic properties on Dowex-1 of authentic phosphorylethanolamine. It also had paper chromatographic properties of phosphorylethanolamine as shown in Figure 6. The second peak obtained from the Dowex-1 column was Pi as determined by paper chromatography and by partitioning radioactivity as the molybdate complex into isobutanol-benzene.

Inorganic phosphate would be expected in these preparations from the action of phosphatase on phosphorylethanolamine. Each of

the fractions contained phosphatase activity as determined by release of Pi from glucose 6-phosphate and release of p-nitrophenol from p-nitrophenyl phosphate.

When the water-soluble ^{32}P was partitioned between water and chloroform as described by Hirschberg et al. (19), negligible radioactivity was detected in the chloroform layer. Since the partition coefficient in this system for phosphoryl sphingosines is approximately 0.73 (19), some radioactivity should have partitioned into the organic phase if phosphoryl sphingosines were present.

Identification of Ceramides as a Product of Ceramide Phosphorylethanolamine Cleavage by Larvae Particulate Preparations

When ^{14}C-labeled ceramide phosphorylethanolamine was incubated with a 10,000-40,000 g particulate fraction, ^{14}C-labeled sphingosine and ^{14}C-labeled phosphorylethanolamine were detected; however, ceramide was not detected in the organic soluble fraction, nor was ^{14}C detected on thin layer chromatograms at the Rf corresponding to ceramide standards. ^{14}C was anticipated in ethanolamine and sphingosine because the ^{14}C-labeled ceramide phosphorylethanolamine was isolated from larvae that were reared in the presence of uniformly labeled L-serine. The data mentioned above indicated that either ceramide was not a reaction product or the preparations contained considerable ceramidase activity. To distinguish between these possibilities, 0.5 mg of tritiated ceramide phosphorylethanolamine–both the sphingosine and fatty acid portion of the substrate contained tritium– was combined with 4 mg of ceramide and incubated with an 8,000-40,000 g fraction. Carrier ceramide was added to dilute any radioactive ceramide that would be produced. The organic soluble reaction products were separated by TLC in three solvent systems, as described in the legend of Table I. Very weak iodine-absorbing spots were detected in the ceramide regions of the thin layer chromatograms although samples equivalent to 1 mg of initial ceramide were applied to the thin layer chromatograms indicating that the particulate preparations contained ceramidase activity. Compounds with Rf's of sphingosine and fatty acids were detected. Although very little, if any, ceramide was detected by iodine vapors on the thin layer chromatograms, some tritium was detected in the ceramide area with the three solvent systems. used. When the tritiated material in the ceramide region of the thin layer chromatograms was eluted and rechromatographed, as described in the legend of Table I, the tritium again migrated with ceramide. Thus, small amounts, approximately 2% (Table I) of the chloroform soluble tritiated material obtained from the reaction media had chromatographic properties of ceramide. Large amounts of sphingosine and fatty acids were produced, presumably from ceramide via the ceramidase activity.

The appearance of 2% of the radioactivity of tritiated ceramide phosphorylethanolamine in the ceramide fraction when carrier ceramide was added to the incubation media is significant, as indicated by the following results. When ^{14}C-labeled ceramide phosphorylethanolamine was incubated with the 10,000-40,000 g particulate fraction in the absence of carrier ceramide, only 0.02% of the initial ^{14}C was detected in ceramide. This amount is 1% of the radioactivity that accumulated in the presence of a large pool of cold ceramide.

Ceramide phosphorylethanolamine might be initially deacylated to sphingosine phosphorylethanolamine and fatty acids, but the data do not indicate such cleavage. ^{32}P-labeled material with thin layer chromatographic properties expected for sphingosine phosphorylethanolamine was not detected in the experiments described for Table I.

DISCUSSION

The data show that a microsome-enriched fraction from housefly larvae converts phosphatidylinositol, phosphatidylserine and phosphatidylglycerol to lysophospholipids and to the respective glycerophosphoryl derivatives. A typical product-precursor relationship was observed for lysophosphatidylinositol (Fig. 4). Previous investigations (8) demonstrated that phosphatidylcholine, phosphatidylethanolamine and phosphatidyl-β-methylcholine are deacylated similarly by microsome-containing preparations. Thus, housefly larvae have the capacity for completely deacylating their principal and minor diacylglycerophosphatides; cardiolipin and phosphatidic acid, both occurring in larvae, have not been investigated. Presumably, this deacylation system can also remove the fatty acids from the numerous abnormal glycerophosphatides that are formed by housefly larvae (20-24).

Formation of glycerophosphorylinositol from phosphatidylinositol occurs in mammalian systems such as ram seminal fluid (25), ox pancreas (26), rat liver (27) and rat prostate (29), and in the microorganism, *penicillium notatum* (26). Phosphatidylinositol is also cleaved by phosphoinositide inositolphosphohydrolase to diglyceride and phosphorylinositol by ram spermatozoa (25), ox pancreas (26), rat liver (27),

guinea pig intestine (28) and guinea-pig brain (40). Our studies indicate that housefly larvae contain little, if any, phosphatidylinositol phosphohydrolase activity. When ^{32}P-labeled phosphatidylinositol was incubated with larval microsomes, no phosphorylinositol was detected in the reaction mixture (Fig. 3B). Some glycerophosphate was found (Fig. 3B), but this undoubtedly was caused by a larval phosphodiesterase that cleaves glycerophosphoryl derivatives (unpublished results) to glycerophosphate and the hydroxyl compound. Phosphorylinositol was not detected when the soluble or particulate fractions were used as the enzyme source. It should be noted that small amounts of ^{32}Pi were detected in some of the reactions. Such results indicate that third instar *M. domestica* larvae contain little, if any, phospholipase C type activity towards phosphatidylinositol.

Ceramide phosphorylethanolamine is a phospholipid in several species of flies (4-6, 30,31) as well as in honey bees (32), scorpions (32), fresh water mollusks (33) and pond snails (34). Our data are consistent with the conclusion that housefly larvae can metabolize ceramide phosphorylethanolamine, as follows:

1. Ceramide phosphorylethanolamine + HOH → ceramide + phosphorylethanolamine
2. Ceramide + HOH → fatty acids + sphingosines

The above-mentioned pathway is supported by the following results:

1. Phosphorylethanolamine was the principal water soluble product when ^{32}P-labeled ceramide phosphorylethanolamine was the substrate. ^{32}Pi was also detected, but it most likely was cleaved from phosphorylethanolamine by a phosphomonoesterase.

2. Tritiated ceramide was detected in the reaction mixture when tritiated ceramide phosphorylethanolamine was the substrate. Tritiated sphingosine and fatty acid were also detected, but these products could be caused by the action of ceramidase on the ceramide.

3. The particulate enzyme preparations contained ceramidase activity. The ceramidase activity was greater than the ceramide phosphorylethanolamine phosphohydrolase activity.

Thus, ceramide phosphorylethanolamine is metabolized by housefly larvae similar to the metabolism of ceramide phosphorylcholine, sphingomyelin, in mammals. In mammals, sphingomyelin is hydrolyzed to ceramide and phosphorylcholine (35,36) and ceramide is cleaved to fatty acids and sphingosines (37,38).

Although phosphatidase C and D activity was not detected with any of the ^{32}P-labeled diacylglycerophospholipids, the metabolism of diacylglycerophosphatides via pathways other than those described above has not been rigorously excluded. Similarly, pathways involving conversion of ceramide phosphorylethanolamine to sphingosine-phosphorylethanolamine or to phosphorylceramide, especially by non-microsome fractions, were not investigated and could occur in housefly larvae.

ACKNOWLEDGMENT

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SHORT COMMUNICATIONS

5α-Cholestan-3β-Ol: High Concentration in Testis of White Carneau Pigeon

ABSTRACT

5α-Cholestan-3β-ol (cholestanol) was isolated from the testes of White Carneau pigeons in the highest concentration thus far reported in any animal tissue. It contributed 26% to 28% of the total sterols (384 ± 29.4 μg cholestanol per gram of wet tissue) in testis, and about 27% of it was esterified. The identity of this stanol was confirmed by $AgNO_3$ thin layer chromatography, gas liquid chromatography and mass spectrometry. Ovaries, liver, plasma and other tissues of this pigeon contained this stanol only to the extent of 2% to 10% of total sterols.

5α-Cholestan-3β-ol (cholestanol) in low concentration is found with cholesterol in all the animal tissues analyzed so far (1). More specific methods (2,3) have revealed that, in guinea pig and rabbit tissues, notably adrenals, cholestanol constitutes as much as 10% to 15% of the total sterols (4,5). This high concentration suggests the possibility of some unknown biological function for cholestanol in these tissues. The testes from these species, however, did not contain much cholestanol. Feeding of cholestanol to animals has resulted in the formation of gallstones (6) and severe atherosclerosis (7) in the thoracic and the abdominal aorta. During our studies with the spontaneously atherosclerosis susceptible White Carneau pigeons, we noted (8) significant excretion of cholestanol in the feces. This finding stimulated us to look for the source of this cholestanol.

White Carneau pigeons (*Columba livia*) were obtained from the Palmetto Pigeon Plant (Sumter, S.C.). All pigeons were adults (four to six years of age). After the pigeons were killed, various tissues were excised and stored at -20 C until analyzed. Free and esterified sterols were extracted from the tissues as a portion of the total lipid extracts (9). Free sterols and their esters were separated from each other and other lipids by thin layer chromatography (TLC) on

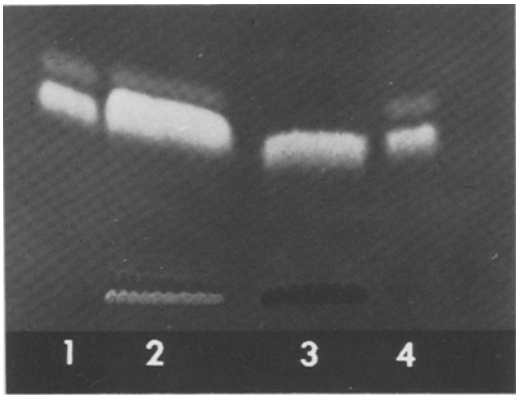

FIG. 1. Thin layer chromatographic separation of sterols, according to degree of unsaturation, on $AgNO_3$-impregnated Silica Gel G. Solvent system, chloroform-methanol-acetic acid, 100:1:0.2 v/v. The plate was stained with 2,7-dichlorofluorescein spray. Numbers 1 and 4, standard mixture of cholestanol (upper band) and cholesterol (lower band); 2, total testis sterols; 3, total liver sterols.

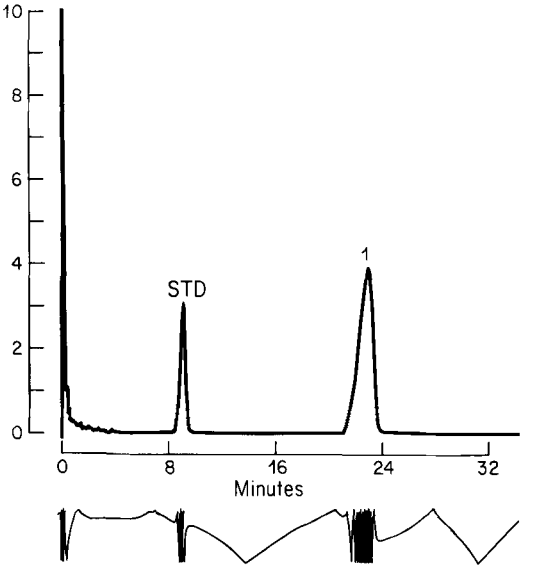

FIG. 2. Gas liquid chromatogram of cholestanol fraction after thin layer chromatography on $AgNO_3$-impregnated Silica Gel G. Chromatographic conditions as described in the text. The stanol was chromatographed as its trimethylsilyl ether (TMsi). STD, cholestane (internal standard); and 1, cholestanol.

TABLE I

Mass Spectrometric Fragmentation Pattern of Cholestanol Isolated From Testis[a]

Principal ions and relative intensities[b]	Interpretation[c]
460 (84.0)	Molecular ion (M)
445 (92.0)	M-15 (methyl group)
370 (35.9)	M-90 (trimethylsilanol group)
355 (52.1)	M-105 (trimethylsilanol + methyl group)
216 (55.9)	M-244 (side chain + ring D + trimethylsilanol)
215 (100)	M-245 (side chain + ring D + trimethylsilanol + H)

[a]Hitachi-Perkin-Elmer RMU-6 D mass spectrometer was used for the analysis. Ion source temperature, 215 C; electron energy, 70 ev; sample pressure, 3.6×10^{-7} mm Hg; inlet temperature, 70 C.

[b]The principal ions of the authentic cholestanol were identical to those of cholestanol isolated from testis. Apart from principal ions listed, both samples contained major peaks at m/e 106, m/e 75, m/e 44 and m/e 69, which seem to have no value in steroid analysis (12). The intensities are expressed relative to the base peak at m/e 215.

[c]Interpretation of fragmentation process is based on reports by Brooks et al. (12) and Eneroth et al. (13).

TABLE II

Cholestanol Content of Some Animal Tissues

Tissue	Total sterol, mg/gm	Cholestanol, %	Reference
Pigeon			
Testis	0.38	27.0	
Adrenals	0.31	4.7	
Ovary	0.06	0.9	This study[a]
Liver	0.20	5.6	
Plasma	2.01	2.2	
Rabbit			
Liver	2.0	4.25	
Small intestine	1.65	5.28	
Adrenals	51.50	8.24	2
Serum	0.29	2.08	
Kidney	3.11	1.97	
Human			
Aorta (intima)	33.8	0.341	2
Aorta (media)	4.79	0.530	
Plasma			
Hypercholesteremic	8.57	1.03	
Pool A	2.34	0.88	2
Pool B	2.51	0.35	
Erythrocytes	1.60	0.32	
Dog			
Serum	1.75	0.56	2
Rat			
Adrenals	0.37	1.1	3
Guinea pig			
Adrenals	3.8	10.1	14
Rooster			
Testis	2.3	0.5	This study[a]

[a]Mean values of duplicate determinations.

Silica Gel G using the solvent system, heptane-isopropyl ether-acetic acid, 65:40:4 v/v (10). After elution with chloroform, the steryl ester fraction was saponified and the sterols were extracted from the saponification mixture (8,11) with ether at an alkaline pH.

The total tissue sterols were fractionated to separate the various 5β-stanols, 3-ketones, Δ^5 sterols, and 5α-stanols as described previously (8). 5β-Stanols and 3-ketones were separated from Δ^5 sterols by TLC using the solvent system, ethyl ether-heptane, 55:45 v/v. Only traces of coprostanol or coprostanone derivatives were present in these tissues. In this solvent system, however, the 5α-stanols (cholestanol) overlap with the Δ^5 sterols (cholesterol). These sterols were separated according to their degree of unsaturation by TLC on $AgNO_3$-impregnated Silica Gel G with the solvent system, chloroform-methanol-acetic acid, 100:1:0.2 v/v (Fig. 1). In the testis sterol fraction there was a strikingly intense band corresponding to cholestanol, while in the fraction from liver this band was very faint; ovary, plasma and all other tissues examined had faint bands in this region. The bands were eluted from the gel and examined by gas chromatography (GLC) as their trimethylsilyl ethers (3.8% W-98 on Diatoport S, 80-120 mesh). An F & M 402 high efficiency gas chromatograph equipped with flame ionization detector was used (operating conditions: column, 230 C; injector, 260 C; detector, 250 C; carrier gas, helium, 50 ml/min). On argentation chromatography, the band corresponding to cholestanol gave a single peak with a retention time corresponding to that of authentic cholestanol (Applied Science Labs, Inc., State College, Pa.), which has a retention time, relative to cholestane, of 2.53 (Fig. 2). In this GLC system, coprostanol and epicholestanol have a retention time less than that of cholesterol (1.89 and 1.93, respectively).

The identity of the cholestanol was further confirmed by mass spectrometry as its trimethylsilyl ether derivative (Table I). The principal ions of fragmentation of cholestanol isolated from the testis were identical to those of authentic cholestanol and agreed with the data of Brooks et al. (12).

The cholestanol and other sterols of varying degrees of unsaturation from various tissues were further quantitated by GLC with cholestane as the internal standard. The cholestanol in the testis of the pigeon contributed about 26% to 28% of the total sterols (384 ± 29.4 μg of cholestanol per gram of wet tissue). This is the highest concentration of cholestanol reported in any animal tissue. Also it should be noted that testis from other species analyzed (3,4) did not contain much cholestanol. About 27% of the cholestanol was present in the esterified sterol fraction. Ovaries contained only about 1% cholestanol. Liver, adrenal, plasma, and other pigeon tissues contained cholestanol only in the range of 2% to 10%. In Table II the cholestanol content of the various pigeon tissues is compared with the data on its distribution in various tissues of other species reported in the literature. It is obvious from this table that the pigeon testis contains the highest proportion of cholestanol. The specific role of cholestanol in the testis of the pigeon should be of interest from the point of view of steroid hormone biogenesis and comparative biochemistry.

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Pristane and Other Hydrocarbons in Some Freshwater and Marine Fish Oils

ABSTRACT

Pristane levels in four commercial freshwater fish oils (alewife, tullibee, maria and sheepshead) were found to be markedly lower (0.0001% or less) than those in marine fish oils (herring, sand launce, cod liver and gray cod liver) selected for comparison on the basis of similar types of depot fat storage (0.008-0.107%). Certain normal alkanes were also identified by gas chromatography. Of these, heptadecane was predominant in all of the freshwater fish oils, but octadecane was more prevalent than heptadecane in three of the marine oils.

Pristane (2,6,10,14-tetramethylpentadecane) occurs ubiquitously in a variety of marine animal lipids, but there appeared to be no records of freshwater fish lipids being examined for this isoprenoid hydrocarbon. Four freshwater fish oils previously studied for fatty acid composition (1-3) have therefore been examined for pristane, phytane (2,6,10,14-tetramethylhexadecane), and a number of associated saturated normal hydrocarbons. The isoprenoid hydrocarbons were essentially absent. For comparison, four marine oils from fish at different trophic levels and from two oceans were analyzed by the same techniques.

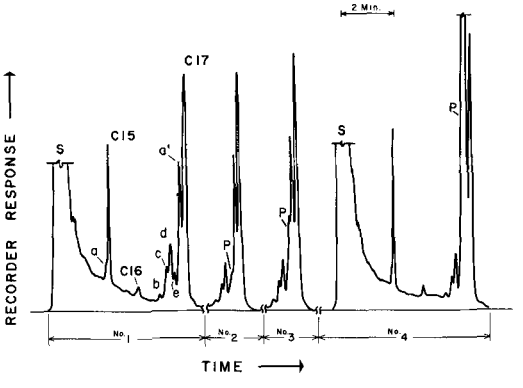

FIG. 1. GLC trace of alewife nonsaponifiable lipid. In traces 2, 3 and 4 pristane (P) has been added in the approximate ratios 1:5:100. Owing to a "load effect" there is a reduction in retention time of pristane (I = 1688 for a low load) until the frontal tangent merges into that of component (e) with I = 1679. S is solvent; C_{15}, C_{16} and C_{17} are normal alkanes and a and a^1 are possible homologs of unknown structure. Analyses on open tubular Apiezon-L column at 190 C with 70 psig helium, operated in a Perkin-Elmer Model 226.

The freshwater fish oils were obtained by processing, on a commercial scale, whole alewife (*Alosa pseudoharengus*), tullibee (*Coregonus artedii*), maria (*Lota lota*) and sheepshead (*Aplodinotus grunniens*). Commercial whole body oils from marine fish were products of Atlantic winter herring (*Clupea harengus*) and Atlantic summer sand launce (*Ammodytes americanus*). The liver oils from Atlantic cod (*Gadus morhus*) and Pacific (gray) cod (*Gadus macrocephalus*) were also of commercial origin.

Quantitative analysis of oils for hydrocarbons by gas liquid chromatography (GLC) was carried out by addition of carbon disulfide containing eicosane in amounts appropriate for an internal standard (0.05-0.2%). A preliminary screening of the oils for hydrocarbons was then carried out by direct analysis of this solution by GLC. The apparatus employed was a Varian–Aerograph Model 600 "Hy-Fi" with flame ionization detector. These analyses were on 3% OV-1 coated on Gas-Chrom Q, packed in a glass column 5 ft long and 1/8 in. o.d., programmed from 150 to 200 C. The peak areas for presumed hydrocarbons were checked on 3% EGSP-Z on Gas-Chrom Q, packed in a stainless steel column 6 ft long and 1/8 in. o.d., programmed from 110 to 180 C. Glass liners in the injection ports (operated at 270 C) were cleaned or replaced daily for this study. Raw oils, without the addition of eicosane, were also examined, but no significant peaks coincided with that of this hydrocarbon in either nonpolar or polar GLC analyses.

Nonsaponifiables were recovered from the oils by an AOCS official method (Ca-6b-53), but without vacuum treatment or taking solutions to dryness. These were examined by GLC on two types of open tubular stainless steel columns, 150 ft long and 0.01 in. i.d. Coatings were either Apiezon-L or butanediol-succinate (BDS) polyester. Apparatus was of Perkin-Elmer manufacture, either Model 226 or Model 900.

In fish, the hydrocarbons, such as pristane, are associated with triglycerides and commercial oils should accurately reflect the original concentration in this lipid as the processing conditions are relatively mild. Total lipid ex-

tracts of fish should give comparable results if sufficient triglyceride is present to dissolve the pristane. Losses of volatile materials are not a problem in handling lipids rich in triglycerides except with prolonged high vacuum treatment at high temperature. However, in our previous work with hydrocarbons in fish (4), it was found that the complete stripping of solvents from small amounts of unsaponifiable materials recovered from fish oils could lead to losses of up to 50% of pristane and other hydrocarbon materials of comparable volatility.

For these reasons an initial GLC examination was carried out on whole oils. The results agreed with the open tubular GLC analysis of the unsaponifiable materials to within ±20%, but this direct technique had serious qualitative inadequacies. Extreme care was required to discriminate between isoprenoid and normal aliphatic components on a purely GLC basis. Thus the Kovats Retention Indices (I) for pristane and phytane were, respectively, Apiezon L, 1688 and 1791, BDS, 1693 and 1800. Only a moderate range of I values are reported for other GLC liquid phases (5,6).

For precise hydrocarbon identifications, the Apiezon-L open tubular column was preferred to the BDS column, but Apiezon-L was subject to a slight "load effect" which altered retention times of interest. This effect is illustrated in Figure 1, where the addition of increments of pristane to alewife nonsaponifiables reduced the retention time (frontal tangent basis) for the increasingly large amount of pristane to that of an unknown component (e) present at 0.001% of oil. It was clear from this type of mixed analysis, carried out on all four freshwater fish oils, that pristane was completely absent from alewife and tullibee oils, and, if present in maria and sheepshead, amounts did not exceed 0.0001% of the whole oil.

All oil samples were scrutinized for phytane, but, at most, only traces were present. Quantitative results (Table I) were taken from Apiezon-L open tubular analyses and trace components (0.0001% or less) could not always be verified on the BDS open tubular analyses because of more obvious baseline irregularities due to liquid phase bleed.

Eicosane was selected as an internal standard since it has nearly the same number of carbon atoms as pristane, although it would in fact be somewhat less volatile, and had a longer retention time than pristane or phytane. The eicosane used (Matheson, Coleman and Bell) contained no detectable octadecane, but did contain 0.04% nonadecane. Appropriate corrections based on recoveries of eicosane were made to the figures for this hydrocarbon in Table I.

TABLE I

Percentages of Selected Hydrocarbons in Some Commercially Produced Oils From Freshwater and Marine Fish

Origin of sample	Species	Identified hydrocarbons, wt %						
		Pristane	Phytane (tentative)	Normal hydrocarbons				
				C_{15}	C_{16}	C_{17}	C_{18}	C_{19}
Lake Michigan	Alewife	<0.0001	0.0002	0.0049	0.0005	0.020	0.0008	0.0001
Lake Winnipeg	Tullibee	<0.0001	0.0002	0.0002	0.0002	0.0065	0.0026	0.0001
Lake Winnipeg	Maria	0.0001	0.0001	0.0004	0.0003	0.0032	0.0014	0.0003
Lake Erie	Sheepshead	0.0001	0.0002	0.0001	0.0001	0.0016	0.0005	0.0001
Atlantic Ocean	Herring	0.107	0.0001	0.0048	0.0010	0.0010	0.017	0.0009
Atlantic Ocean	Sand launce	0.052	0.0003	0.0005	0.0008	0.0010	0.0062	0.0010
Atlantic Ocean	Cod liver	0.0084	0.0001	0.0001	0.0001	0.0008	0.0002	0.0014
Pacific Ocean	Grayfish Liver	0.014	0.0001	0.0007	0.0011	0.0010	0.017	0.0007

Most of the oils produced minor peaks (about 10% of the total GLC recorder area) which were not directly identifiable. Only the alewife contained as yet unidentified major components (Fig. 1, a and a[1]). Coupled GLC and mass spectrometry constitute virtually the only technique applicable to the absolute confirmation of structures of minor components for which standards may not be available (6-8).

The marine species (herring, sand launce) feeding most directly on herbivorous crustacea have the highest level of pristane (Table I). In the marine environment the bulk of the observed pristane is apparently formed from phytol by copepods (9,10). These are a principal food of herring, but are widely eaten by all smaller predators. The alfewife is perhaps the most comparable freshwater species in respect to feeding habits and fat deposition in the body, and has also the highest levels of phytanic acid and of total isoprenoid fatty acids among the four freshwater oils (3). The maria, like the cod, store depot fat in their livers, but the fat content for whole fish was low (3.7% vs. 8-12% for the other three) and the unsaponifiable material in the oil high (4.1% vs. 1% for the other three) for these particular samples of freshwater fish (1). There are apparently no comparable literature results for pristane in freshwater fish species, but other teleost marine fish data include 0.22% (7) and <0.035% (11) for herring oil, 0.14% for the mackerel *Scomber scombrus* (12), 0.003% for cod liver oil (7), and 0.02-0.03% for eulachon (*Thaleichthys pacificus*) lipid (4). There is no known reason for seemingly low values for the fish liver oils, but slow metabolic oxidation to pristanic acid (13) might be promoted by liver fat storage as distinct from body storage.

In general, the biochemical conversion of phytol to isoprenoid fatty acids in the freshwater environment is not known to differ from that occurring in the marine environment (3). The direct conversion of phytol to pristane by freshwater invertebrates, if it occurs at all, must be very much less effective than in the marine environment. Marine algae are reported to contain pristane, while normal hydrocarbons are fairly common in algae from both environments (14-17). In the alewife, the origin of the high levels of pentadecane and heptadecane (Table I) and of the as yet unidentified pair of possible related components, a and a[1] (Fig. 1), may be from freshwater algae. The latter unknowns were not significant in the other freshwater fish oils or in the marine oils.

Different processes for the origin of pristane could operate in specific fashions in the various large freshwater lakes. Thus, the formation of pristane during the decay of phytol by microbial or other processes in lake bottoms could be a factor of possible interest and importance. This would be different from lake to lake and from comparable turnover in the oceans, depenent on factors of depth, bottom surface relative to water volume, and quantity and type of biomass. Fish can assimilate fat soluble materials, such as chlorinated pesticides, from their environment by exchange across the gills as well as through dietary intake (18). Pristane and phytane would probably share with these compounds the two important properties of relative inertness to metabolic degradation by fish and of a very high degree of solubility in fats. The relative absence of pristane and phytane from three major freshwater lakes indicates either little production of isoprenoid hydrocarbons or facile biological destruction of the amounts which are produced.

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Detection of a Sebacate Contaminant From Chloroform[1]

ABSTRACT

One of the major contaminants of reagent grade chloroform is identified as a diester of sebacic acid. The contaminant is not present in redistilled chloroform.

It is standard practice in lipid laboratories to redistill even reagent grade solvents to free them of contaminants. The contaminant problem was considered in detail by Rouser et al. (1). In this report, a major contaminant in reagent grade chloroform is identified as a diester of sebacic acid.

The amount of residue found in chloroform after evaporation at 40 C was determined to be 2.5 mg (0.00042%). The residue was chromatographed on thin layer chromatography having an Rf slightly greater than triolein. The compound was eluted and saponified in 0.5 N NaOH in methanol and methylated with BF_3-methanol. The gas liquid chromatography analyses of the resulting methyl esters were made on a Barber-Colman Model 10 Gas Chromatograph equipped with a radium 226 detector source. A 3 m x 6 mm glass column packed with DEGS on Gas Chrom A (Applied Science Laboratories) and a 3 m x 6 mm glass column of 10% DEGA (2% H_3PO_4) on Gas Chrom A (Applied Science Laboratories) were maintained at 170 C. The unknown peak had a retention time of 12.3 min on the DEGS column which was slightly less than that for methyl stearate, retention time 12.5 min. On the DEGA column the peak had a retention time of 14.1 min which was slightly less than methyl-heptadecanoate, retention time 14.7 min.

An IR spectrum was recorded on a Perkin-Elmer Model 137 Infracord Spectrophotometer. The eluate, analyzed as a thin film on a KBr pellet, gave major absorptions at 2960 cm^{-1} ($-CH_2-$), 1720 cm^{-1} (C//O), and 1280 cm^{-1} (C//O -O-R). The total eluate was also chromatographed on a column of 3% SE-30 on Chromosorb WHP 80/100 mesh (Supelco Inc.) at a temperature of 230 C. The eluate contained a single peak which was analyzed on an LKB-9000 gas chromatograph-mass spectrometer using a column packed with SE-30 as described and at identical temperature. An ionization current of 70 eV was employed. The mass spectrum had a large peak at m/e 185. This fragment is suggested to be the protonated anhydride formed by the cleavage of a diester of sebacic acid. The fragment at m/e 112 may represent the dehydrated fragment of octyl alcohol. The fragment at 314 corresponds to the loss of one octyl group from a dioctyl ester of sebacic acid. A standard di(2-ethyl hexyl) sebacate was obtained from a collection of common plasticizers. Di(2-ethyl hexyl) sebacate gave identical retention times and mass spectra to the contaminant isolated from chloroform (Fig. 1).

Dimethyl sebacate was prepared by methylating sebacic acid as previously described. The dimethyl sebacate had identical

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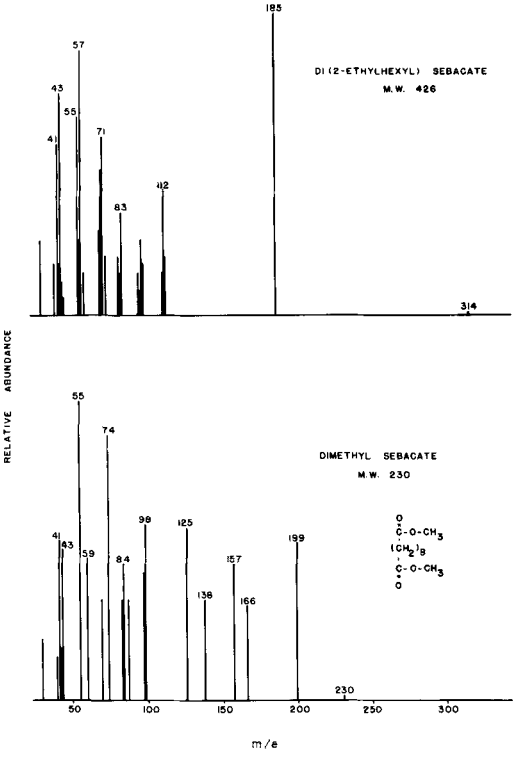

FIG. 1. (Top) Mass spectrum of the chloroform contaminant eluted from a TLC plate. (Bottom) Mass spectrum of the peak appearing in the fatty acid methyl esters.

retention times and an identical mass spectrum to the methylated compound isolated from chloroform. When 400 ml of chloroform was redistilled in glass, no residue was obtained.

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Stereospecific Analysis of Maize Triglycerides

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ABSTRACT

This is the first report of stereospecific analyses of plant triglycerides isolated from seeds of distinct genotypes rather than from commerically refined oils. Triglycerides from six maize inbreds were analyzed. The strains exhibited a wide range of fatty acid compositions (palmitic acid 7.8-19.1%, oleic acid 17.0-43.0%, linoleic acid 41.6-68.3%). The distribution of the fatty acids among the 1, 2 and 3 positions of the triglycerides was clearly nonrandom for all six strains. At the 2 position of the triglycerides over 98% of the fatty acids were unsaturated. More palmitic and stearic acids were found in position 1 than in 3. The general fatty acid pattern of maize triglycerides was similar to that found in most animal triglycerides.

INTRODUCTION

Methods (1,2) have recently been developed to determine the composition of the fatty acids esterified in positions 1, 2 and 3 of triglycerides [The stereospecific numbering system approved by the IUPAC-IUB Commission on Biochemical Nomenclature (3) has been used throughout this paper.]. Brockerhoff's procedure (1) involves the preparation of random diglycerides by hydrolysis of the triglycerides with pancreatic lipase. Phosphatidyl phenols are synthesized from the resulting diglycerides. These phospholipids are hydrolyzed with the stereospecific phospholipase A of snake venom. As only 1,2-diacyl-phosphatidyl phenol is hydrolyzed by the enzyme, the fatty acid composition of the resulting lysophosphatide is that of the 1 position of the original triglycerides. The composition of the 2 position is determined by pancreatic lipase hydrolysis of the triglycerides. The fatty acids of the 3 position are then calculated by subtracting the data for the 1 and 2 positions from the total triglyceride.

Many animal triglycerides (4-13) have been subjected to stereospecific analysis. In animals the distribution of fatty acids at the three positions was not random, but rather each position had a characteristic fatty acid composition.

Very few plant triglycerides (8,14,15) have been stereospecifically analyzed. There is still some question as to whether the distribution of fatty acids is or is not random between the 1 and 3 positions of triglycerides from plants. In the previous structural studies of maize and other plant triglycerides, only commercially refined oils were used. It is unlikely that commercial oils are representative of the triglycerides as they are synthesized in the plants. The source materials are genetically heterogeneous, and the oils might undergo structural modification during processing. Stereospecific analyses of plant triglycerides should be conducted on pure breeding strains of genetically defined backgrounds.

Maize offers many advantages over animal tissue for biosynthetic studies of triglycerides. There are no complicating influences from exogenous dietary lipids. Maize triglycerides have a relatively simple fatty acid composition. Sampling can be done on homogeneous materials at the same physiological state of maturity. The genetic diversity that exists among maize strains permits biosynthetic studies over a wide range of fatty acid distributions. This paper describes the stereospecific analyses of triglycerides from six strains of maize.

MATERIALS AND METHODS

Preparation of Triglycerides

All of the maize strains were grown at the Agronomy farm of the University of Illinois at Urbana, Illinois during the summers of 1967, 1968 and 1969. The ears were hand-pollinated. At maturity the corn was harvested and dried to approximately 10% moisture.

Kernels were removed from the middle portion of each ear and ground in a Spex ball mill. The meal (approximately 12 g) was extracted three times with 15 ml portions of petroleum ether (bp 60-68 C). The sample was agitated under nitrogen 30 sec with a Vortex mixer during the extraction. After centrifugation the petroleum ether extract was decanted. The solvent was reduced in a rotary flash evaporator.

The triglycerides were isolated by thin layer chromatography (TLC). The lipids (approximately 50 mg per thin layer plate) were streaked with a semi-automatic sample appli-

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cator (Supelco, Inc., Bellefonte, Pa.) on plates coated with a 0.5 mm layer of Silica Gel G (E. Merck, Darmstadt, W. Germany). The plates were developed in a nitrogen atmosphere with petroleum ether-diethyl ether-acetic acid (80:20:1).

The triglycerides were located with transmitted light, scraped from the plate, and extracted from the silica gel with 10 ml of diethyl ether-methanol (9:1). The silica gel and solvents were vortexed for 15 sec under nitrogen and centrifuged. The supernatant was decanted. This extraction step was repeated twice, and the supernatants were combined and evaporated in a flash evaporator vented with nitrogen.

Prevention of Autoxidation

The accuracy of the stereospecific analyses was greatly improved when very careful attention was paid to the prevention of autoxidation. Whenever possible, the lipids were handled in a nitrogen atmosphere. Thin layer plates were run in a nitrogen atmosphere and sprayed after development with BHT [0.02% butylated hydroxy-toluene in chloroform-methanol (1:1)]. BHT (10 μl of 0.1% solution) was added to the isolated triglycerides and in every subsequent step.

Preparation of Methyl Esters and GLC Analysis

Known weights of methyl heptadecanoate were added to samples of the triglycerides as internal standards. Methyl esters of the triglycerides were prepared with boron trifluoride-methanol according to a modified Morrison and Smith procedure (16). The lipids were transmethylated in a 12 ml vial sealed with Teflon cap liner and tape. The vial was heated in an oven at 102 C for 50 min. Water (1 ml) was added, and the methyl esters were extracted with two 2 ml portions of petroleum ether.

The conditions for gas chromatography of the fatty acid methyl esters have been described previously (17).

Preparation of the Diglycerides

Diglycerides were prepared by pancreatic lipase hydrolysis of the triglycerides. The commercial lipase preparation (Steapsin, General Biochemicals, Chagrin Falls, Ohio) contained traces of lipids. The fatty acids of these lipids were mainly palmitic and stearic acids. Extraction of the steapsin at room temperature twice with acetone (5:1 v/wt) and twice with anhydrous diethyl ether (5:1) (18) removed the contaminating lipids.

Finer, more stable suspensions of the lipase were obtained when histidine was added to the enzyme stock mixture. The lipase (10 mg/ml) was suspended in 0.01 M histidine at pH 7.0 (19).

The procedure used for the lipase hydrolysis was a modification of that of Lands et al. (2). The triglycerides (30 mg), dissolved in petroleum ether, were pipetted into a 50 ml centrifuge tube, and the solvent was removed with a stream of nitrogen. The following reagents were added: 1.8 ml of 1.0 N NaCl, 1.2 ml of 1.0 M tris HCl (pH 8.0), 0.3 ml of 0.11 M $CaCl_2$ and 0.3 ml of lipase. The reaction was carried out under nitrogen. The components were agitated on a Vortex mixer at room temperature for 3 min and then the enzymatic hydrolysis was stopped with 0.4 ml of 3 N HCl. The lipids were extracted with 10 ml of $CHCl_3$: CH_3OH (2:1) and with 10 ml of $CHCl_3$. The combined chloroform extracts were dried over anhydrous Na_2SO_4 for 1 hr, filtered through glass wool, and evaporated to dryness in a flash evaporator.

The lipids were dissolved in 0.1 ml of $CHCl_3$:CH_3OH (2:1) and streaked on a 0.5 mm TLC plate. The plate was developed with petroleum ether-diethyl ether-acetic acid (65:35:1). The diglycerides were visualized by transmitted light and extracted with diethyl ether-methanol (9:1) twice and $CHCl_3$/CH_3OH/H_2O (50:45:5) once. An aliquot was methylated to check that random diglycerides had been produced. The monoglyceride band was located by spraying the plate with 2,7-dichlorofluorescein (0.1% in ethanol). The monoglycerides were extracted from the silica gel with the same solvents as the diglycerides. GLC of the methyl esters of the monoglycerides determined the fatty acid composition at the 2 position of the original triglycerides.

Preparation of Phosphatidyl Phenols From Diglycerides

The Brockerhoff procedure (15) was modified in the following manner. The mixture was shaken during the 90 min reaction time, since this increased the yield of phosphatidyl phenols. The R_f of the phosphatidyl phenols was 0.55 on a 0.3 mm TLC plate developed with a solvent system of $CHCl_3$:CH_3OH:NH_4OH (85:15:2) (8). The phosphatidyl phenols were located with transmitted light and extracted from the silica gel with $CHCl_3$:CH_3OH:H_2O (50:45:5) twice and CH_3OH once.

Phospholipase A Hydrolysis of the 1,2-Diacyl-Phosphatidyl Phenol

Ether (1 ml) was added to the phosphatidyl phenols, followed by 7.5 ml of 0.1 M aqueous triethylammonium bicarbonate at pH 7.5 (triethylamine in water saturated with CO_2), two

drops of 0.05 M $CaCl_2$, and 2 mg of snake venom from king cobra (*Ophiophagus hannah*) (Sigma Chemical Co., St. Louis, Mo.). *Ophiophagus hannah* venom was used instead of *Crotalus atrox* venom as suggested in the original procedure (15). King cobra venom does not show preferential hydrolysis of individual fatty acids (20).

After the reaction had proceeded for 24 hr under nitrogen, the mixture was transferred to a rotary flash evaporator flask with chloroform-methanol (1:1). Acetic acid (two drops) was added and enough isobutanol to layer (to prevent foaming). The mixture was evaporated under reduced pressure and finally under high vacuum. The residue was dissolved in $CHCl_3/CH_3OH$ (1:1), transferred to a test tube and centrifuged. The supernatant was applied to 0.3 mm TLC plates which were developed in petroleum ether-diethyl ether-acetic acid (50:50:1) (9). The bands were detected by transmitted light. The phospholipids, which remained near the origin in this solvent system, were recovered and rechromatographed in $CHCl_3/CH_3OH/NH_4OH$ (85:15:2). Both the lysophosphatidyl phenol and 2,3-diacyl-phosphatidyl phenol were isolated and methylated with boron trifluoride-methanol for fatty acid analysis by gas chromatography.

RESULTS AND DISCUSSION

The 1,3 random, 2 random theory of fatty acid distribution (21,22) has been applied to corn and other vegetable oils. Support for this theory has been based on fractionation of the triglycerides by $AgNO_3$-TLC and lipase hydrolysis. The experimental data obtained for corn oil (23,24) agreed well with the values calculated by the 1,3 random, 2 random theory. Since commercial corn oil had been used in these studies, it seemed that triglycerides from pure maize strains should be tested.

The concentrations of the triglyceride species obtained from $AgNO_3$-TLC (25) of the inbreds, NY16, H59 and C103, are listed in Table I. The completely random distribution was calculated directly from the fatty acid composition in the original triglyceride. The restricted 1,3 random, 2 random distribution was calculated as described by Coleman (22) from lipase data. The completely random and the 1,3 random, 2 random distributions were in close agreement, and both models closely fit the actual concentrations obtained by $AgNO_3$-TLC. Probability values from chi-square analyses of greater than 0.90 were obtained for all three strains. We were unable to differentiate statistically between the completely random

TABLE I

Concentrations of the Major Triglyceride Species From the Maize Inbreds, NY16, C103 and H59

Species	Percent of total triglyceride[a]									
	NY16			C103			H59			
	Observed	Random	1,3 Random 2 Random	Observed	Random	1,3 Random 2 Random	Observed	Random	1,3 Random 2 Random
S_2M[b]	0.3	0.4	0.3	2.5	2.6	1.7	2.3	3.5	1.9
SM_2	1.3	0.9	1.1	8.9	7.7	7.4	3.8	3.5	3.3
S_2D	1.0	1.3	1.0	3.4	2.4	3.0	7.1	6.1	6.4
M_3	2.2	1.0	1.0	11.2	8.3	8.2	3.0	1.5	1.5
SMD	6.7	6.8	6.8	16.3	14.7	16.2	16.4	15.9	17.2
M_2D	10.6	9.6	9.7	21.4	24.2	24.4	8.7	10.3	10.5
SD_2	13.5	11.4	11.7	7.2	7.2	8.1	19.9	17.7	19.8
MD_2	28.0	32.0	32.0	21.1	24.4	23.8	20.7	23.1	22.9
D_3	34.7	35.1	35.0	7.2	7.7	7.0	16.8	17.2	15.5
D_2T	1.5	1.5	1.4	0.8	0.8	0.8	1.4	0.9	0.9

[a]Analyses done in triplicate for all three inbreds.
[b](Abbreviations:) S, saturated; M, monoene; D, diene; T, triene fatty acids.

TABLE II

Precision of Stereospecific Analysis of Triglycerides From Maize Inbred, H51

	Compound or position	Obtained from	Fatty acid distribution, mole %[a]				
			16:0	18:0	18:1	18:2	18:3
I	Triglyceride		17.7 ± 0.7	1.8 ± 0.1	31.2 ± 0.3	48.1 ± 0.4	1.2 ± 0.2
II	*α,β*-Diglyceride	Calc. $\frac{3xI + V}{4}$	13.6	1.4	30.1	53.7	1.2
III	PPh[b]		13.6 ± 1.1	1.7 ± 0.1	30.2 ± 0.3	53.1 ± 0.8	1.4 ± 0.1
IV	1	LysoPPh	26.0 ± 1.1	3.4 ± 0.5	30.8 ± 1.4	38.8 ± 0.9	1.0 ± 0.2
V	2	Monoglyceride	1.5 ± 0.5	0.1 ± 0.1	26.8 ± 0.3	70.6 ± 0.2	1.0 ± 0.1
VI	3	Calc. 3xI-IV-V	25.4	2.0	36.1	34.9	1.6
VII	3′	Calc. 2xVIII-V	24.4	3.0	36.9	35.3	0.4
VIII	2,3-PPh		12.9 ± 0.8	1.5 ± 0.1	31.9 ± 0.3	53.0 ± 0.5	0.7 ± 0.3

[a]The experimental values shown are the means of four separate determinations ± standard deviation.

[b]Phosphatidyl phenols (PPh) before phospholipase A treatment; should agree with calculated *α,β*-diglycerides.

TABLE III

Stereospecific Analyses of Triglycerides From Five Maize Strains[a]

Strain	Compound or position	Fatty acid distribution, mole %				
		16:0	18:0	18:1	18:2	18:3
NY16	TG[b]	7.8	1.9	20.7	68.3	1.2
	1	15.6	3.9	21.4	57.8	1.3
	2	0.7	0.2	21.6	76.6	0.8
	3	7.0	1.6	19.2	70.6	1.6
IRLO	TG	12.5	0.9	17.0	67.9	1.7
	1	21.5	2.1	15.8	59.0	1.6
	2	1.1	0.1	16.3	81.0	1.5
	3	14.9	0.6	18.8	63.6	2.1
M14	TG	12.5	1.2	28.9	56.2	1.1
	1	25.3	2.8	28.2	42.6	1.1
	2	1.2	0.2	31.1	66.5	1.0
	3	11.0	0.7	27.3	59.7	1.3
H59	TG	19.1	1.6	23.0	54.9	1.3
	1	30.3	3.0	18.8	46.2	1.8
	2	1.2	0.1	20.2	76.9	1.6
	3	25.8	1.8	30.1	41.6	0.6
C103	TG	12.5	2.2	43.0	41.6	0.6
	1	22.4	4.2	41.2	31.7	0.5
	2	1.0	0.3	40.4	57.5	0.8
	3	14.2	2.1	47.5	35.6	0.6

[a]Analysis done in quadruplicate for all strains.
[b]TG, triglyceride.

and the 1,3 random, 2 random models. The completely random and the 1,3 random, 2 random models may be statistically indistinguishable because the saturated fatty acid content of corn triglycerides is too low. Since the saturated fatty acids are almost completely restricted from the 2 position, they should show the greatest deviation from randomness. The saturated acids ranged from 7.6% for NY16 to 19.0% for H59 in these three strains. Deviations have been detected with highly saturated vegetable fats such as cocoa butter and akee oil (24).

A different analytical method had to be employed to determine whether the distribution of fatty acids was random or nonrandom. The Brockerhoff procedure (15) for stereospecific analysis was used to examine the compositions of the fatty acids esterified at the 1, 2 and 3 positions of the maize triglycerides.

One of the requirements for accurate stereospecific analysis is that the fatty acid distribution of the α,β-diglycerides must be the same as the distribution in the original triglycerides. If nonrepresentative diglycerides are used for preparation of the phosphatidyl phenols, the fatty acid compositions determined for position 1 and calculated for position 3 of the original triglyceride will not be reliable.

Pancreatic lipase hydrolysis should give representative diglycerides, since maize triglycerides do not contain significant amounts of the long chain polyunsaturated fatty acids which are resistant to lipase (26). However, Anderson et al. (27) have pointed out that some pancreatic lipase procedures do not remove the fatty acids randomly from the 1 and 3 positions even from corn oil and other vegetable oils with relatively simple fatty acid distributions. It is necessary to determine the diglyceride composition after each lipase hydrolysis and compare it with the expected composition. This was done for all the maize strains analyzed. In Table II an example of this comparison is shown for maize inbred, H51. The fatty acid composition of the phosphatidyl phenols prepared from the experimental diglycerides was compared to the calculated composition of the α,β-diglycerides. Differences less than 2% absolute were obtained with our lipase hydrolysis procedure.

In the stereospecific analysis a second independent determination of the fatty acids at the 3 position may be made by isolating the 2,3-diacyl-phosphatidyl phenols after the phospholipase A hydrolysis (28). The agreement of these two calculations of position 3 (Table II) indicates the accuracy achieved by the procedure. Analyses with differences less than 5% for major components were accepted. For the majority of the maize strains analysed the agreement was much closer than this.

The results of stereospecific analyses of the

triglycerides from six maize strains are given in Table II and Table III. The distribution of the fatty acids among the 1, 2 and 3 positions of the triglycerides was clearly nonrandom. The general fatty acid pattern for maize triglycerides was similar to that found in animal triglycerides (2,4,8,11). The saturated fatty acids, palmitic and stearic acids, were predominately esterified at the 1 position. The 2 position contained the most linoleic and the least saturated acids. In the 2 position over 98% of the fatty acids were unsaturated. A higher percentage of saturated fatty acids was found in position 1 than in position 3 and the difference in 3 was made up by oleic acid or linoleic acid or both.

The percentage of oleic acid was higher in position 3 than in the other two positions of the triglycerides of all the corn strains analyzed except NY16 and M14 where it was slightly higher in 1. In four of the strains, NY16, Illinois Reverse Low Oil (IRLO), M14 and C103, linoleic was higher in position 3 than in 1, but in H51 (Table II) and H59, the order was reversed. These changes did not follow concentration differences in 18:1 and 18:2 in the total triglycerides. Instead, the variation in positional specificities found among these inbred maize strains indicated genetic control of fatty acid placement.

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Effects of Fatty Acid Concentration and Positional Specificity on Maize Triglyceride Structure

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ABSTRACT

The effects of fatty acid concentration and positional specificity on maize triglyceride structure were evaluated from the stereospecific analyses of triglycerides from 12 genotypes. The fatty acids at each position were influenced by the fatty acid concentration in the total triglyceride except for the saturates in the 2 position. The fatty acid concentration had the greatest effect on the fatty acid composition of position 3. The existence of positional specificity was evident from the nonrandom distribution of the fatty acids among the three positions of the triglycerides. The concentration and positional specificity effects could be separated in selected genotypes and their crosses. This indicated different genetic controls for each effect.

INTRODUCTION

Triglycerides isolated from kernels of inbred maize lines have been stereospecifically analyzed (1). Each position showed a distinct composition of fatty acids. However, for oleic acid and linoleic acid there was no consistent pattern of asymmetry among the maize strains. In some strains oleic was higher in position 3 than in 1; in others it was higher in 1 than in 3. Similar results were obtained for linoleic acid. The concentrations of these fatty acids in the total triglyceride seemed to have some influence on their distribution among the three positions of the triglycerides, but there also appeared to be positional specificity.

In this paper the effects of these two factors, concentration of fatty acids in the total triglyceride and positional specificity, have been examined. The range of fatty acid compositions in maize and the possible genetic combinations offer unique material for studying these effects.

MATERIALS AND METHODS

The source of materials and the methods employed in this paper are described in a companion report (1).

RESULTS

The concentration effect is defined as the influence of the amount of a particular fatty acid available for esterification. The effect of fatty acid concentration in the total triglyceride on each triglyceride position was assessed from stereospecific analyses of twelve maize strains. These strains exhibited a very wide range in fatty acid distributions (saturated acids 9.7-20.9%, oleic acid 17.0-53.9%, linoleic 32.6-68.3%).

In Figure 1 the concentrations of saturated acids (S), monoene (M) and diene (D) in the total triglyceride are individually correlated with their respective concentrations at the 1 position. Highly significant correlations of 0.86,

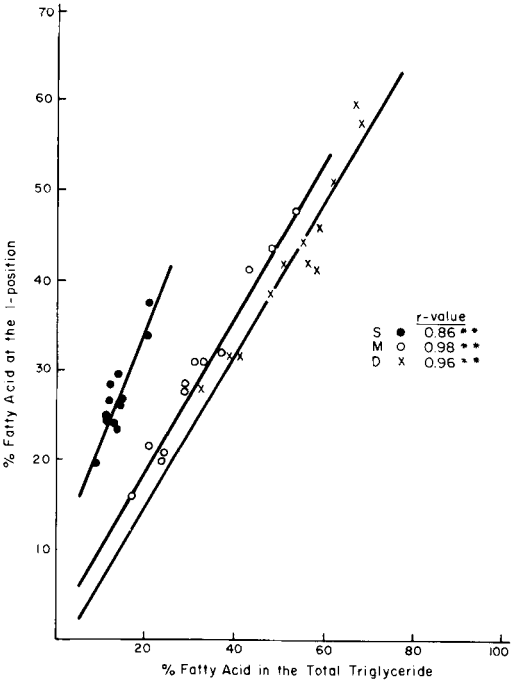

FIG 1. The concentration of S, M and D in the 1 position compared with total triglyceride. S, M and D denote saturated, monoene and diene fatty acids, respectively.

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TABLE I

Statistical Parameters for the Relationship Between Fatty Acids in Total Triglyceride and Fatty Acids at Each Position

Fatty acid	Position in triglyceride	$\bar{x}$	r[a]	a[c]	b[d]
16:0 + 18:0	1	26.6	0.86[b]	+ 9.95	1.19
	2	1.0	0.57	+ 0.11	0.07
	3	14.3	0.92[b]	- 10.30	1.76
18:1	1	29.9	0.98[b]	+ 1.36	0.88
	2	29.4	0.96[b]	+ 1.65	0.86
	3	38.0	0.96[b]	- 3.03	1.26
18:2	1	42.7	0.96[b]	- 1.48	0.83
	2	68.7	0.94[b]	+24.66	0.81
	3	46.0	0.95[b]	- 28.89	1.41

[a]r Correlation coefficient.
[b] Significant at the 1% level.
[c]a y intercept.
[d]b Regression coefficient.

0.98 and 0.96 were obtained for S, M and D, respectively (Table I).

Correlation coefficients were also calculated for M and D in the total and in the 2 position and found to be significant (r=.96 and .94, respectively) (Fig. 2 and Table I). When S in the total was correlated with S at the 2 position, a nonsignificant r value of 0.57 was obtained. Apparently, the concentration of S in the total triglyceride had little influence on the amount of S esterified at the 2 position. This is expected, because there was almost complete restriction of S from the 2 position.

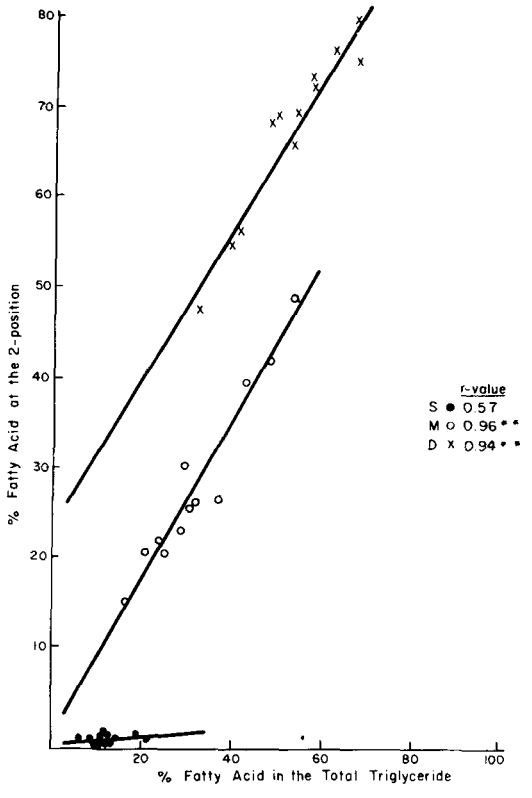

FIG 2. The concentration of S, M and D in the 2 position compared with total triglyceride.

The S, M and D fatty acids in the 3 position were all positively correlated with the total (Fig. 3 and Table I). This indicates that all three fatty acid types exert a significant concentration effect at the 3 position of the triglyceride.

A comparison of regression slope values for all three positions (Table I) suggests that the fatty acid composition at position 3 is the most strongly influenced by the concentration of S, M and D in the total triglycerides. We also observed that the least amount of positional specificity was exerted at this position (1).

In the maize triglycerides each fatty acid showed a concentration effect at each position except for the saturates at the 2 position. Strong specificity against S at this position resulted in a nonsignificant concentration effect. Concentration effects can only be present when the fatty acids exhibit partial or no positional specificity.

Positional specificity of fatty acid among the three positions of triglyceride could result from different rates of esterification. If these differences are major, then significant deviations from a "completely random" distribution would be found. All 12 maize strains showed a nonrandom distribution of fatty acids.

Thus, it is concluded that fatty acid distribution in maize triglycerides is the result of two major effects, concentration and positional

specificity. If fatty acid synthesis and the positional specificity of esterification are under different genetic controls in maize, one may expect to find strains with differing positional specifications and the same fatty acid composition (positional specificity effect). Conversely, it may be possible to find strains with the same positional specificities and different fatty acid compositions (concentration effect).

Analyses of individual kernels from C105 for fatty acid composition revealed the presence of considerable variation in oleic and linoleic acids. It far exceeded the usual environmental variability of homozygous strains. Individuals showing extremes in linoleate content were selfed either one or two generations. Two homozygeous strains phenotypically identical except for a 6.4% difference in linoleate were isolated. The two strains were subjected to stereospecific positional analysis (Table II). The fatty acid distributions of all three positions were different, but the proportions of each fatty acid (notably oleic and linoleic) were similar at each position. Since proportion is an expression of positional specificity, it is concluded that these two strains of C105 were genetically similar for positional specificity.

A hybrid with C105 as the maternal parent was detected that had oleic and linoleic contents similar to those of Illinois High Oil (IHO).

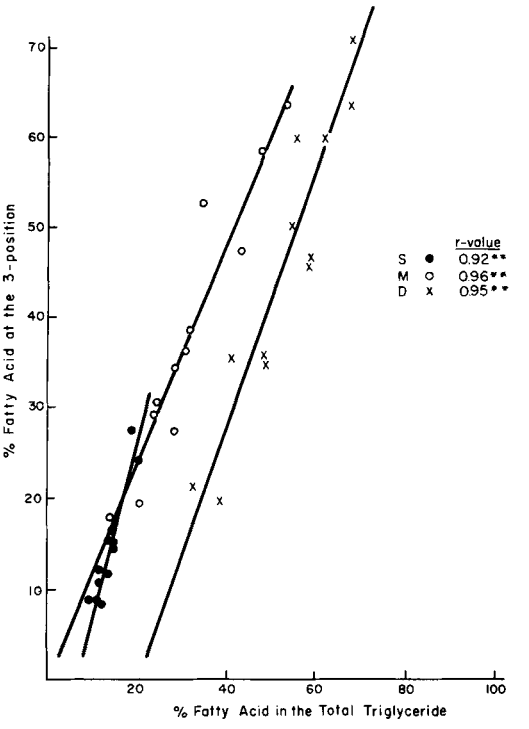

FIG 3. The concentration of S, M and D in the 3 position compared with total triglyceride.

TABLE II

Stereospecific Analyses of Triglyceride From Two Strains of C105

Compound or position	Fatty acid distribution, mole per cent[a]				
	16:0	18:0	18:1	18:2	18:3
Strain A					
Triglyceride	10.6	2.3	53.9	32.6	0.6
1	19.9	3.9	47.9	27.9	0.4
Per cent in 1[b]	62.6	56.5	29.6	28.5	22.2
2	0.6	---	50.0	48.9	0.4
Per cent in 2	1.9	---	30.9	50.0	22.2
3	11.3	3.0	63.8	21.0	1.0
Per cent in 3	35.5	43.5	39.5	21.5	55.6
Strain B					
Triglyceride	10.2	2.0	48.4	39.0	0.4
1	20.2	4.1	43.7	31.5	0.5
Per cent in 1	66.0	68.3	30.1	29.4	41.7
2	0.5	---	42.9	56.0	0.6
Per cent in 2	1.6	---	29.5	52.3	50.0
3	9.9	1.9	58.6	19.5	0.1
Per cent in 3	32.4	31.7	40.4	18.2	8.3

[a]Analyses done in triplicate.

[b]Per cent of fatty acid that is esterified at this position = $\frac{\%\text{ of fatty acid in this position} \times 100}{\%\text{ of fatty acid in triglyceride} \times 3}$

TABLE III

Stereospecific Analyses of Triglycerides From Two Maize Strains With Similar Fatty Acid Distributions

Compound or position	Fatty acid distribution, mole per cent				
	16:0	18:0	18:1	18:2	18:3
F_1[a]					
Triglyceride	8.6	1.8	37.6	51.3	0.8
1	21.4	3.9	35.0	39.1	0.6
Per cent at 1	82.9	72.2	31.0	25.4	33.3
2	0.9	0.1	33.2	65.2	0.6
Per cent at 2	3.5	1.9	29.4	42.4	33.3
3	3.8	1.4	44.6	49.6	0.6
Per cent at 3	14.7	25.9	39.5	32.2	33.3
IHO[b]					
Triglyceride	12.2	1.8	36.2	49.2	0.6
1	22.6	3.4	31.9	41.8	0.3
Per cent at 1	61.7	63.0	29.4	28.3	16.7
2	0.8	---	27.4	71.1	0.7
Per cent at 2	2.2	---	25.2	48.2	38.9
3	13.2	2.0	49.3	34.7	0.8
Per cent at 3	36.1	37.0	45.4	23.5	44.4

[a]F_1 hybrid with C105 as the maternal parent. Analysis done in duplicate.
[b]Analysis done in triplicate.

TABLE IV

Stereospecific Analyses of Reciprocal Crosses of Two Maize Inbreds

Strain	Compound or position	Fatty acid distribution, mole per cent				
		16:0	18:0	18:1	18:2	18:3
NY16 x C103[a]	Triglyceride	10.3	1.6	28.6	58.6	0.8
	1	22.3	3.9	27.4	45.5	0.9
	Per cent at 1	72.1	80.4	31.9	25.9	35.7
	2	0.9	0.3	24.2	73.9	0.8
	Per cent at 2	2.8	5.8	28.2	42.0	31.4
	3	7.8	0.7	34.3	56.5	0.8
	Per cent at 3	25.1	13.8	39.9	32.1	32.9
C103 x NY16	Triglyceride	10.3	1.7	32.1	55.1	0.8
	1	21.3	3.2	30.4	44.3	0.8
	Per cent at 1	68.8	65.1	31.5	26.8	30.1
	2	0.6	0.2	27.6	70.8	0.7
	Per cent at 2	2.0	4.4	28.7	42.9	28.9
	3	9.0	1.5	38.4	50.1	1.0
	Per cent at 3	29.2	30.5	39.8	30.3	41.0

[a]Analyses done in quadruplicate.

The stereospecific data (Table III) show the proportions of oleic and linoleic acids at positions 2 and 3 to be quite different in each strain. A far greater percentage of linoleate was at the 2 position of IHO as compared to the C105 F_1 hybrid. Further, the order of preference of linoleate between positions 1 and 3 was reversed in these two strains. Oleate had a stronger preference for the 3 position of IHO triglycerides as compared to the other strain. These positional differences were all significant. Thus, these two strains possess essentially the same concentrations of oleic and linoleic acids in their total triglycerides but differ in their positional specificities of esterification.

The triglycerides of the reciprocal crosses of two maize inbreds, NY16 and C103, were stereospecifically analyzed (Table IV). An LSD test was used to test the statistical significance of the data. The concentrations of oleic and linoleic acids in the total triglycerides of the two reciprocal crosses were significantly different at the 5% level. Linoleic acid was higher in NY16 x C103 than in C103 x NY16, while oleic acid was higher in C103 x NY16. However, the proportions of these fatty acids at all three positions were not significantly different. The two reciprocal crosses show identical positional specificities.

The effect of fatty acid concentration was clearly shown when the triglyceride species of the reciprocal crosses (Table V) were isolated by silver nitrate thin layer chromatography (2). The percentages of the triglyceride species which contained two or three moles of linoleic acid were higher in NY16 x C103 than in C103 x NY16. In contrast, those species containing two or three moles of oleic acid were higher in C103 x NY16. Although the positional specificities of the two crosses were identical, the fatty acid composition of the total triglyceride had a very strong influence on the amounts of the various triglyceride species.

TABLE V

Concentrations of Triglyceride Species of Reciprocal Crosses of Two Maize Inbreds

Species	Per cent of total triglyceride	
	NY16 x C103[b]	C103 x NY16
S_2M[a]	1.1 ± 0.3	1.2 ± 0.3
SM_2	4.6 ± 1.3	4.6 ± 0.2
S_2D	2.4 ± 0.4	2.0 ± 0.3
M_3	3.5 ± 0.2	5.7 ± 0.1
SMD	12.2 ± 0.2	12.2 ± 0.2
M_2D	12.9 ± 0.2	16.3 ± 0.2
SD_2	13.9 ± 0.5	11.8 ± 0.8
MD_2	26.4 ± 1.4	26.5 ± 1.7
D_3, D_2T	22.5 ± 0.9	19.6 ± 1.0

[a]Abbreviations: S, saturated; M, monoene; D, diene; T, triene fatty acids.
[b]Mean of four samples ± standard deviation.

DISCUSSION

Several conclusions can be drawn from positional specificity and concentration effects in maize triglycerides. In the 12 strains studied the proportions of oleate and linoleate were most constant in the 1 position. In other words, positional specificity at the 1 position was very uniform over a wide range of oleate and linoleate composition. All the fatty acids exhibited only partial specificities at all three positions with the exception of the saturated acids at position 2. Thus, with one exception, each position showed a sizeable concentration effect for each fatty acid. The concentration effects for oleate and linoleate were very similar at positions 1 and 2 of the triglycerides (similar regression line slopes, Fig. 1 and 2 and Table I). The fatty acids at the 3 position were the most strongly influenced by the content of saturated acids, oleate and linoleate in the total triglyceride.

The specific location of fatty acids in the triglyceride molecules of vegetable oils is important for several reasons. First, the placement of the fatty acids in the triglycerides affects their stability toward oxidation. When Sahasrabudhe and Farn (3) heated corn oil, they found that the fatty acids in the 1 and 3 positions were more susceptible to oxidation than those in the 2 position. Raghuveer and Hammond (4) suggested that concentrating the unsaturates at the 2 position may orient the triglyceride molecule to make it more stable against autoxidation.

High specificity for polyunsaturated acids at the 2 position is also desirable from a nutritional point of view. From 72% to 80% of the dietary triglycerides are absorbed into the intestinal wall as 2-monoglycerides (5). The free fatty acids are either metabolized or reesterified. The monoglycerides are converted to triglycerides and polar lipids. Since polyunsaturated fatty acids are essential in the diet, esterification of these acids at the 2 position would ensure their conservation during digestion.

If we better understood both the biochemical regulation of fatty acid synthesis and the mechanisms for placement of these acids in the triglyceride molecules of plants, triglycerides could be produced for specific purposes.

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The Selective Utilization of Diglyceride Species Into Maize Triglycerides

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ABSTRACT

The maize triglycerides were resolved into species by silver nitrate thin layer chromatography. The distribution of the fatty acids among the 1, 2 and 3 positions of each triglyceride species was determined by stereospecific analysis. From these data the relative amounts of each positional isomer were calculated. The results indicate that esterification of the fatty acids at each position proceeds with a specificity that is correlated with the composition of the other positions of the triglyceride.

INTRODUCTION

The previous papers (1,2) showed that the distribution of fatty acids in the three positions of maize triglyceride was nonrandom. Furthermore, esterification of fatty acids into each position of glycero-3-phosphate was a function of both the concentration of available fatty acid substrates and the fatty acid specificity indigenous to each position. The concentration effect was by far the most significant and its presence tended to obscure positional specificity. Stereospecific examination of individual triglyceride species would allow a critical evaluation of positional specificity by diminishing the effect of concentration. The purpose of this investigation was to isolate selected triglyceride species from two inbreds of maize and to subject these species to stereospecific analysis.

EXPERIMENTAL PROCEDURES

The inbreds, H59 and C103, were selected for this study. Triglycerides were prepared as described previously (1) except that a 20 g sample of seed from each strain was extracted. The final yield of total triglyceride for both lines was approximately 600 mg.

Thin layer chromatography (TLC) plates were coated 0.3 mm thick with 12% silver nitrate in Silica Gel G (E. Merck, Darmstadt, W. Germany). The plates were dried in the dark for 30 min, activated at 102 C for 90 min and stored in the dark in a desiccator over $CaCl_2$. Twenty milligrams of triglyceride were streaked on each plate with a semiautomatic sample applicator (Supelco, Inc., Bellefonte, Pa.). The methanol-chloroform system used to develop the plates varied from 0.6-2% methanol in chloroform, depending on absorbent thickness, temperature and humidity. The triglyceride bands were detected under UV light after spraying the plate with a 0.1% ethanolic solution of 2,7-dichlorofluorescein. A 0.02% solution of butylated hydroxy-toluene (BHT) in $CHCl_3$:CH_3OH(1:1) was sprayed on each plate to prevent autoxidation. Selected bands were scraped from the plate into test tubes. The triglyceride was extracted from the silica gel with 5 ml of diethyl ether-methanol (9:1). The

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TABLE I

Comparison of the Fatty Acid Composition of Original M14 Triglycerides With the Combined Methyl Esters From Individual Triglyceride Classes

Triglycerides and combined methyl esters	Fatty acid composition, relative area per cent				
	16:0	18:0	18:1	18:2	18:3
Original triglyceride[a]	12.2	1.1	26.0	59.6	1.1
Plate 1 Combined methyl esters	12.2	1.1	25.9	59.8	1.1
Plate 2 Combined methyl esters	12.8	1.3	26.5	58.1	1.3

[a]Mean of two samples.

TABLE II

Comparison of the Observed and Calculated Fatty Acid Distributions of 2,3-Phosphatidyl Phenols From the Maize Inbred, H59

Class		Fatty acid distribution, mole per cent S[a]	M	D
SMD	Obs.	24.8	37.2	38.0
	Cal.[b]	22.5	37.8	39.5
M_2D	Obs.	4.4	58.0	37.6
	Cal.	0.5	61.6	37.8
SD_2	Obs.	29.0	1.3	69.8
	Cal.	27.5	2.3	70.3
MD_2	Obs.	1.6	36.1	62.3
	Cal.	0.6	36.2	63.2

[a]Abbreviations: S, saturated; M, monoene; D, diene fatty acids.

[b]Calculated 2,3-phosphatidyl phenol = $\frac{\text{2-position} + \text{3-position}}{2}$.

mixture was vortexed for 15 sec under a stream of nitrogen and then centrifuged, and the supernatant decanted. The extraction was repeated twice. The supernatants were combined and dried under a stream of nitrogen.

The SMD, M_2D, SD_2 and MD_2 species obtained from the 15 preparative TLC plates were combined. Each fraction represented from 10-25 mg of triglyceride. Stereospecific analyses of duplicate samples were performed as described previously (1).

RESULTS AND DISCUSSION

Gunstone and Padley (3) have expressed skepticism as to whether a proportional extraction of all triglyceride species can be obtained from the silver nitrate-silica gel complex. They suggested that the highly unsaturated triglycerides may be more difficult to extract. To determine if the extraction of the maize triglyceride species was proportional, two 15 mg samples of triglyceride from inbred M14 were fractionated by silver nitrate TLC. The 10 bands were extracted. Methyl esters were prepared from each band and then combined. The fatty acid composition of the combined fractions was determined and compared with the original M14 triglyceride (Table I). It is apparent that the fatty acid composition of the combined methyl esters from individual triglyceride classes agrees very closely with the original triglyceride. Thus, this elution procedure results in a proportional extraction of each triglyceride species.

In the stereospecific analysis, the fatty acid composition of the 2,3-phosphatidyl phenol

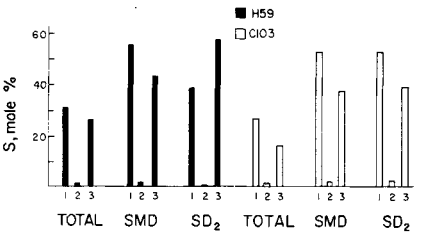

FIG 1. The distribution of saturated fatty acids at each position of total and two species of H59 and C103 triglycerides.

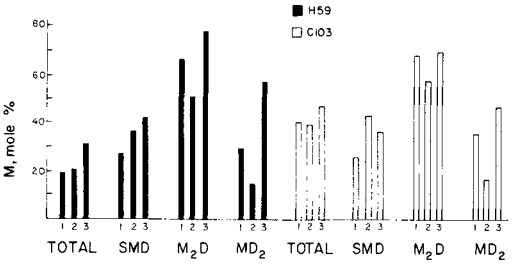

FIG 2. The distribution of oleate at each position of total and three species of H59 and C103 triglycerides.

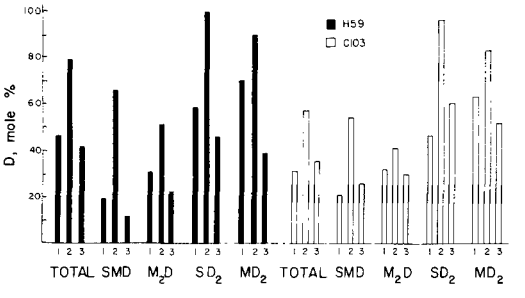

FIG 3. The distribution of linoleate at each position of total and four species of H59 and C103 triglycerides.

isomer can be used to check the accuracy of the fatty acid distributions determined for the 2 and 3 positions of the triglycerides. The observed and calculated fatty acid distributions of 2,3-phosphatidyl phenol from the maize inbred, H59, are given in Table II. The calculated fatty acid distribution of the 2,3 isomer of phosphatidyl phenol was obtained from the observed fatty acid distributions at the 2 and the 3 positions. This data is representative of the experimental precision obtained from the combined techniques of silver nitrate TLC fractionation and stereospecific analysis.

Figure 1 shows the distribution of the saturated fatty acids at the three positions of total, SMD and SD_2 triglycerides from H59 and

TABLE III

Concentrations of H59 and C103 Triglyceride Species

Triglyceride species	Per cent of total triglyceride	
	C103[b]	H59
S_2M[a]	2.5	2.3
SM_2	8.9	3.8
S_2D	3.4	7.1
M_3	11.2	3.0
SMD	16.3	16.4
M_2D	21.4	8.7
SD_2	7.2	19.9
MD_2	21.1	20.7
D_3	7.2	16.8
D_2T	0.8	1.4

[a]Abbreviations: T, triene fatty acids; see also Table II.

[b]Analyses done in triplicate.

C103. The percentages of saturated fatty acids in the total triglycerides of H59 and C103 are higher at the 1 position than at the 3 position. Only small amounts of S are esterified at the 2 position. This same profile was obtained for SMD from H59 and for SMD and SD_2 from C103. In contrast, the SD_2 species from H59 has more S at the 3 than at the 1 position. Thus, the saturated fatty acids differ in their distribution among species within the same strain of maize. Differences also occurred for the same species in different strains.

The distribution of oleate at each position of the total, SMD, M_2D, and MD_2 triglycerides of H59 and C103 is given in Figure 2. The monoene is preferred in the 3 position in the total triglyceride and in all the species of H59 and C103 except SMD from C103. In the latter, M is preferred at the 2 position. The M_2D and MD_2 species of H59 and C103 show a preference for M at the 1 position over the 2 position. In contrast, in the SMD species in these two strains M prefers the 2 position over the 1 position.

The distributions of D at the 1, 2 and 3 positions of total, SMD, M_2D, SD_2 and MD_2 triglyceride from H59 and C103 are summarized in Figure 3. Linoleate is principally in the 2 position of all these fractions. In H59, D prefers the 1 position over the 3 position, although the ratio varies among these five fractions. In C103, D is preferred at the 3 position over the 1 position of total, SMD and SD_2. On the other hand, the M_2D and MD_2 species from C103 closely resemble the H59 results.

These data clearly demonstrate that individual fatty acids behave differently in the various triglyceride species. Thus, certain fatty acid

TABLE IV

Determination of Correlative Specificity for C103 and H59 Triglycerides

Triglyceride ratio	C103	H59
$\frac{SDM}{SDD}$[a]	1.2	0.8
$\frac{DSM}{DSD}$	1.0	2.0
$\frac{DDM}{DDD}$	1.4	0.8
$\frac{DMM}{DMD}$	1.9	0.9
$\frac{MDM}{MDD}$	1.1	0.3
$\frac{MMM}{MMD}$	1.9	1.5
$\frac{M_{III}}{D_{III}}$[b]	1.3	0.6

[a]Abbreviations: see Tables II and III.

[b]M and D fatty acid distribution at 3 position of total triglyceride.

combinations are preferred over others, indicating an interaction of fatty acids among the different positions. Further, these positional differences between the C103 and H59 strains for the same species imply a genetic basis for these variations.

In the de novo synthesis of triglyceride and phospholipids via the α-glycerophosphate pathway, it has been shown that 1,2-diglyceride is the common precursor of triglyceride, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (4). However, structural analyses of these lipids from rat liver revealed differences at the 1 and 2 position of all three lipids. The fatty acids esterified at the 1 position of PC and PE were the same, but different from the 1 position of triglyceride; the 2 position differed in all three lipids (5).

Selective utilization of diglyceride species for the synthesis of these three lipids could explain the differences. Wood and Harlow (5) showed that some pairing of the fatty acids or selection of certain diglyceride species was probably occurring during the biosynthesis of PC, PE and triglyceride in rat liver. When Elovson et al. (6,7) studied the incorporation of intraportally injected (^{3}H)-glycerol into rat liver glycerolipids, their data also indicated a selective utilization of diglyceride in the synthesis of PE, PC and TG.

In contrast, Lands and co-workers (8,9), using rat liver slices, found that all diglyceride species were esterified to triglycerides at a

similar rate, i.e., acylation of the diglycerides proceeded with noncorrelative specificity. However, the diglyceride and its phosphatidic acid precursor had very asymmetrical fatty acid distributions (10,11). They suggested that nonrandom synthesis of species of diglycerides was occurring, followed by an almost random utilization of the various diglyceride species for the biosynthesis of triglycerides.

When Christie and Moore (12) analyzed triglycerides from a variety of pig tissues, specific pairing of particular fatty acids was found in the triglycerides of some tissues more than others. This selectivity was greatest in the liver and blood triglycerides, less in the adipose tissue triglycerides and quite low in the milk triglycerides. These differences may reflect the influence of diet on triglyceride biosynthesis in animals. Plant triglyceride biosynthesis would not be affected by this complicating factor.

The data from the stereospecific analyses of SMD, M_2D, SD_2 and MD_2 species of H59 and C103 were evaluated to determine whether the diglyceride species were being selectively incorporated into maize triglyceride. The relative amount of each positional isomer present in each species was determined from the fatty acid distribution at each position of that species and from the percentage composition of all 10 species (Table III). These data were then tested for correlative specificity according to the procedure of Slakey and Lands (8). The concentrations of the SD, DS, DD, DM, MD and MM diglyceride species were determined and compared with the M/D ratio at position 3 of each triglyceride species (Table IV). If triglyceride synthesis proceeds with noncorrelative specificity (i.e., random utilization of DG), the triglyceride species ratios should be the same, irrespective of the diglyceride species. Clearly, these ratios are different in both the H59 and C103 strains. Thus, esterification of fatty acids at the 3 position proceeds with a specificity that is correlated with the composition of the other two positions of the molecule. These data are consistent with the selective utilization of diglycerides for triglyceride biosynthesis that was observed by Wood and Harlow (5) and Elovson et al. (6) in rat liver tissue.

Limited data obtained on C103 triglyceride species suggest that the fatty acid compositions at the 1 position and the 2 position are interrelated. Species containing either M or D at position 1 were compared with the M/D ratio at position 2. The results below suggest that correlative specificity may also be operating between these two positions.

$$\frac{MMS^* + MMM + MMD}{MDS + MDM + MDD} = 1.1$$

$$\frac{DMS + DMM + DMD}{DDS + DDM + DDD} = 0.7$$

(*Stereospecific analysis was also performed on the SM_2 triglyceride species from C103.)

The data obtained in this series of three papers add further support to the proposal of similar pathways of triglyceride biosynthesis in animals and higher plants. It has been demonstrated that the distribution of fatty acids is nonrandom in all three positions of plant triglycerides as it is in the triglycerides of animals. Maize and animal triglycerides show similar positional specificities, i.e., S mostly at 1, D mostly at 2 and M mostly at 3. This suggests similar substrate specificities and enzymatic systems. Finally, the demonstration that diglycerides are selectively utilized in the synthesis of plant triglycerides agrees with recent results obtained with animal tissue.

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Oleate Hydratase: Studies of Substrate Specificity

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ABSTRACT

This work concerns studies of the substrate specificity of an enzyme preparation from a pseudomonad which catalyzes the stereospecific hydration of the Δ^9-double bond of oleic acid. [5DL-^{3}H]-5DL-hydroxystearic acid, [6DL-^{3}H]-6DL-hydroxystearic acid, [7DL-^{3}H]-7DL-hydroxystearic acid, [9DL-^{3}H]-9DL-hydroxystearic acid, [10DL-^{3}H]-10DL-hydroxystearic acid, [11DL-^{3}H]-11DL-hydroxystearic acid, [14DL-^{3}H]-14DL-hydroxystearic acid, [15DL-^{3}H]-15DL-hydroxystearic acid, and [1-^{14}C]-stearolic acid were prepared and incubated with the enzyme. Only the 10-hydroxystearic acid served as a substrate for the enzyme. The findings reported in this study, in conjunction with those previously reported, indicate that only the D-isomer of 10-hydroxystearic acid serves as a substrate for the enzyme.

INTRODUCTION

In 1962, Wallen, Benedict and Jackson (1) described the isolation of a pseudomonad which efficiently converted added oleic acid to 10-hydroxystearic acid. We have shown that the 10-hydroxystearic acid formed in this system is optically active (2,3) and has the D (or R) configuration (3). Subsequent studies with the intact organism in a medium enriched with deuterium oxide indicated that the formation of 10D-hydroxystearic acid from oleic acid proceeds with the incorporation of one atom of stably bound deuterium at carbon atom 9 in the L (or R) configuration (4,5). The successful preparation of a soluble (105,000 x *g*) extract of the organism which catalyzes the reaction in question (6,7) permitted further studies of the mechanism of the reaction. The enzyme preparation catalyzed the interconversion of oleic acid and 10D-hydroxystearic acid (7). The 10-hydroxystearic acid formed in the reaction was shown to be optically active and to have the D (or R) configuration (7). Evidence compatible with a mechanism involving a hydration of the double bond has been presented (7). The crude enzyme preparation also catalyzed the formation of Δ^{10}-*trans*-octadecenoic acid from either oleic acid or 10D-hydroxystearic acid (7). The enzyme also catalyzed the conversion of palmitoleic acid to 10-hydroxypalmitic acid (7) and the conversion of linoleic acid to 10D-hydroxy-Δ^{12}-*cis*-octadecenoic acid (8). 9D-Hydroxystearic acid did not serve as a substrate for the enzyme (7). Catalysis, by the enzyme, of the stereospecific hydration of 9,10-*cis*-epoxystearic acid to yield one isomer of threo-9,10-dihydroxystearic acid and of the stereospecific hydration of 9,10-*trans*-epoxystearic acid to yield one isomer of erythro-9,10-dihydroxystearic acid was observed (9,10).

In the present work we have prepared [5DL-^{3}H]-5DL-hydroxystearic acid, [6DL-^{3}H]-6DL-hydroxystearic acid, [7DL-^{3}H]-7DL-hydroxystearic acid, [8DL-^{3}H]-8DL-hydroxystearic acid, [9DL-^{3}H]-9DL-hydroxystearic acid, [10DL-^{3}H]-10DL-hydroxystearic acid, [11DL-^{3}H]-11DL-hydroxystearic acid, [14DL-^{3}H]-14DL-hydroxystearic acid, and [15DL-^{3}H]-15DL-hydroxystearic acid. Upon incubation of these labeled acids with the bacterial enzyme, only the 10-hydroxystearic acid served as a substrate. Upon repeated incubation of the labeled 10-hydroxystearate, utilization of only ~45% of the racemic substrate was observed, a finding compatible with a utilization of only one of the two enantiomers of 10-hydroxystearic acid.

An analogy exists between the enzyme under consideration and the enzyme fumarate hydratase. Both enzymes catalyze the stereospecific hydration of olefinic double bonds. Recently, we have shown that fumarate hydratase also catalyzes the stereospecific hydration of L-*trans*-2,3-epoxysuccinate to yield mesotartrate (11,12). Teipel, Hass and Hill (13) have shown that fumarate hydratase catalyzes the hydration of acetylene dicarboxylate to yield oxaloacetate. To investigate the possibility that the bacterial enzyme might hydrate the acetylenic acid corresponding to oleate, we have prepared [1-^{14}C]-stearolic acid. Upon incubation of the labeled acid with the enzyme, significant formation of 10-ketostearate (or 9-ketostearate) could not be demonstrated.

EXPERIMENTAL PROCEDURES AND RESULTS

Materials and Methods

9D-Hydroxystearic acid was a sample pre-

pared previously (3). 10D-Hydroxystearic acid was prepared as outlined previously (3). Samples of 5-, 6-, 7-, 8-, 11-, 14- and 15-hydroxystearic acids were provided by B. Sammuelsson. An authentic sample of stearolic acid was a gift of K. Bloch. Sodium borotritide and [1-^{14}C]-oleic acid were purchased from the New England Nuclear Corporation. The ^{14}C-labeled oleic acid was purified, in the form of the methyl ester, by chromatography on an activated silicic acid column. The radiopurity of the oleate was judged to be in excess of 99% on the basis on thin layer chromatographic analysis on a Silica Gel G plate (solvent system, petroleum ether-ether-acetic acid, 90:10:1). The petroleum ether used in these studies had a boiling range of 30 to 60 C.

Methods employed for the measurement of radioactivity, recording of melting points, thin layer radiochromatographic analyses, gas liquid radiochromatographic analyses, measurement of protein concentration, and preparation of methyl esters have been described previously (7,14). The preparation of the soluble bacterial enzyme and conditions of incubation have also been described previously (7).

Preparation of [10DL-^{3}H]-10DL-Hydroxystearic Acid

To methyl 10D-hydroxystearate (156 mg) in acetic acid (1.0 ml) was added chromium trioxide (38 mg) in 90% acetic acid (0.38 ml). After 2.3 hr at room temperature, water was added and the resulting mixture was extracted three times with petroleum ether. The pooled extracts were washed with water and dried over anhydrous sodium sulfate. The residue (155 mg) obtained on evaporation of the solvent was applied to a silicic acid column (5 g; Merck). Using a 20% ether in petroleum ether as the eluting solvent, fractions 6 ml in volume were collected. The contents of fractions 1 through 6 were pooled and recrystallized from petroleum ether-acetone, yielding methyl 10-ketostearate (130 mg; mp 49-51 C). The product showed a single component on thin layer chromatographic (TLC) analysis on a Silica Gel G plate (solvent: petroleum ether-ether-acetic acid, 70:30:1).

To methyl 10-ketostearate (3.48 mg) in methanol (0.5 ml) was added sodium borotritide (1.0 mCi; 02.6 mg) in 0.9 N NaOH (1.1 ml). After standing 5 hr at room temperature, the mixture was acidified and extracted with ether. The residue obtained upon evaporation of the solvent was dissolved in methanol (0.3 ml) and 1 N NaOH (0.2 ml) was added. After standing 3 hr at room temperature, the mixture was acidified and extracted with ether. The residue obtained upon evaporation of the solvent was applied to a silicic acid column. After the passage of petroleum ether through the column to elute traces of the methyl ester, the labeled 10-hydroxystearic acid (1.55 mg) was eluted with ether. The radiopurity was judged to be in excess of 99% on the basis of thin layer radiochromatographic analysis. The specific activity was ~2.5 x 10^{7} cpm per mg.

Preparation of [9DL-^{3}H]-9DL-Hydroxystearic Acid

Methyl 9-ketostearate (105 mg; mp 48.5-50.5 C; single component on TLC analysis on a Silica Gel G plate) was prepared from methyl 9D-hydroxystearate (139 mg) as described above in the case of the methyl 10-ketostearate. Reduction of the methyl 9-ketostearate with sodium borotritide yielded [9DL-^{3}H]-9DL-hydroxystearate which was purified as described in the case of the reduction of the 10-ketostearate. The isolated acid had a specific activity of 2.11 x 10^{7} cpm/mg and a radiopurity in excess of 99% as judged by thin layer radiochromatographic analysis.

Preparation of Tritium-Labeled 5-, 6-, 7-, 8-, 11-, 14-, 15- and 16-Hydroxystearic Acids

The methyl esters of each of the racemic 5-, 6-, 7-, 8-, 11-, 14-, 15- and 16-hydroxystearic acids (from 1.4 to 2.6 mg) were prepared by treatment with diazomethane. The methyl esters were dissolved in methanol (0.2 ml) and subjected to chromic acid oxidation as in the case of the 10-hydroxystearate. Each of the ketostearates were dissolved in methanol (0.5 ml) and sodium borotritide (0.9 mCi; 0.22 mg) in 0.09 N NaOH (0.1 ml) was added. After standing at room temperature for 10 hr, 1 N NaOH (0.15 ml) was added and the resulting mixtures were left at room temperature for 3 hr. The labeled acids were isolated and purified as described above for the tritium-labeled 10-hydroxystearic acid. The radiopurity of each of the 7-, 8-, 11-, 14-, 15- and 16-hydroxystearic acids was judged to be in excess of 99% on the basis of thin layer radiochromatographic analysis on a Silica Gel G plate. In the case of the [5-DL-^{3}H]-5DL-hydroxystearic acid another labeled component, faster-moving on TLC analysis, was noted. This component, presumably the lactone, accounted for 33% of the recovered radioactivity. A similar observation was made with the labeled 6-hydroxystearic acid, although in this case the faster-moving component accounted for 15% of the radioactivity. The crude [^{3}H]-5DL-hydroxystearic acid was applied to a silicic acid column (3 g) in a 10% ether in petroleum ether and the column was successively eluted with the same solvent mixture (200 ml), 30% ether in petro-

TABLE I

Enzymatic Utilization of Racemic 10-Hydroxystearic Acid

Incubation	Extent of conversion of 10*DL*-hydroxystearate to monounsaturated acids	Cumulative utilization of 10*DL*-hydroxystearic acid[a]
1	7.4	7.4
2	5.7	12.7
3	7.2	19.0
4	5.0	23.0
5	6.2	27.8
6	5.8	32.1
7	5.5	35.8
8	4.0	38.3
9	3.8	40.7
10	2.6	42.2
11	1.0	42.8
12	1.7	43.8
13	0.8	44.2
14	0.3	44.5

[a]A brief note is included to indicate the method of calculation of this value. After the first incubation (7.4% conversion) the recovered, labeled 10-hydroxysterate was reincubated with the enzyme and 5.7% of the added substrate was converted to monounsaturated fatty acid (incubation 2). This represents a cumulative per cent utilization of 12.7% [0.057 (100-7.4)].

leum ether (200 ml), and ether (300 ml). Fractions 5 ml in volume were collected. The labeled 5-hydroxystearic acid, eluted in fractions 49 through 116, showed a single radioactive component on thin layer radiochromatographic analysis. The labeled 6-hydroxystearic acid was also purified by silicic acid column chromatography as described above in the case of the labeled 5-hydroxystearic acid.

Preparation of [1-^{14}C]-Stearolic Acid

Bromine (52 mg) was added dropwise with stirring to methyl [1-^{14}C] oleate (~90 μCi; 96 mg) in petroleum ether (5 ml) which was maintained at 4 C (15). The petroleum ether solution was washed twice with 5 ml portions of a 5% sodium thiosulfate solution and three times with 5 ml portions of water. The petroleum ether solution of the dibromide was dried over anhydrous sodium sulfate and yielded a colorless oil (161 mg) upon evaporation of the solvent.

Dehydrobromination was effected by heating the dibromide under reflux with KOH (86 mg) in ethylene glycol (2 ml) for 6 hr (16). After cooling the reaction mixture to room temperature, a dilute solution of HCl was added. The resulting mixture was extracted three times with 10 ml portions of petroleum ether. The combined extracts were washed with water and dried over anhydrous sodium sulfate. The crude product was applied to an activated silicic acid column (21 x 2.3 cm) and the product (76 mg) was eluted as a single peak with a 10% ether in petroleum ether. Upon thin layer radiochromatographic analysis on a Silica Gel G plate (solvent system, petroleum ether-ether-acetic acid, 70:30:1) the radioactive product showed the same chromatographic mobility as authentic stearolic acid. The radiopurity was judged to be at least 98.7% on the basis of this analysis. Gas liquid radiochromatographic analysis of the methyl ester on a 5% diethylene glycol succinate column showed a single radioactive peak with the same retention time as that of authentic methyl stearolate. The specific activity was ~1.9 x 10^6 dpm/mg.

Incubation of [10DL-^{3}H]-10DL-Hydroxystearic Acid With Enzyme

[10DL-^{3}H]-DL-Hydroxystearic acid (6.09 x 10^6 cpm) was dissolved in 0.02 N KOH (0.2 ml) and incubated with the soluble enzyme preparation (8 ml; 75 mg protein) for 3 hr at 10 C. Methanolic KOH (5 ml; 1.8 N; 90% methanol) was added, and the resulting mixture was heated on a steam bath for 30 min. After cooling to room temperature, the mixture was acidified to pH 1 and extracted three times with ether. The combined ether extracts were washed with water and dried over anhydrous sodium sulfate. The extent of conversion of the hydroxy fatty acid to monounsaturated fatty acids (oleic acid plus Δ^{10}-*trans*-octadecenoic acid) was assayed by thin layer radiochromatography on Silica Gel G plates (solvent: petroleum ether-ether-acetic acid, 70:30:1) on an aliquot of tht ether solution and found to be 7.4%. The hydroxy and monounsaturated fatty acids were separated on an activated silicic acid

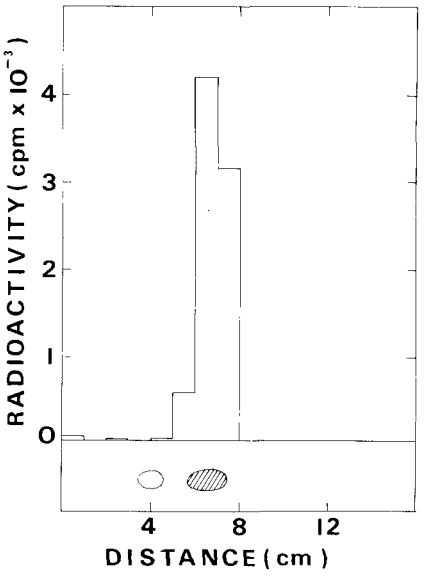

FIG. 1. Thin layer radiochromatographic analysis of products of incubation of [1-^{14}C]-stearolic acid with the bacterial enzyme. ○, 10-ketostearic acid; ●, stearolic acid.

column (3 g), using 10% ether in petroleum ether (200 ml) to elute the monounsaturated acids and 50% ether in petroleum ether(200 ml to elute the 10-hydroxystearic acid. The recovered labeled 10-hydroxystearic acid was reincubated with the enzyme preparation, and the incubation mixture was worked up as described above. A total of 14 incubations were carried out. The results are listed in Table I. After 12 incubations of the racemic substrate with the enzyme, only slight additional conversion of the hydroxystearate to monounsaturated acids was observed. After a total of 14 incubations the cumulative percent utilization of the racemic substrate was approximately 45%. Since we have previously shown that the enzyme catalyzes the formation of oleate (and Δ^{10}-*trans*-octadecenoic acid) from the D-enantiomer of 10-hydroxystearic acid (7) and that the 10-hydroxystearate formed in the enzyme-catalyzed hydration of the double bond of oleate had the D-configuration (7), the finding of only ~50% utilization of the racemic hydroxystearate is compatible with a specific utilization of the D-enantiomer of 10-hydroxystearate.

Incubation of [9DL-^{3}H]-9DL-Hydroxystearic Acid With Enzyme

[9DL-^{3}H]-9DL-Hydroxystearic acid (1.9 x 10^{6} cpm) was dissolved in 0.02 N KOH (0.15 ml) and incubated with the soluble enzyme preparation (5 ml; 47 mg protein) for 2.3 hr at 10 C. The incubation mixture was treated as described in the case of the labeled 10-hydroxystearate. No conversion (<0.1%) of the labeled 9-hydroxystearate to monounsaturated fatty acid was observed.

Incubation of Labeled 5-, 6-, 7-, 8-, 11-, 14-, 15-, and 16-Hydroxystearic Acids With Enzyme

Each of the labeled hydroxyacids (15-500 mμmoles) was dissolved in 0.02 N KOH (0.1-0.2 ml) and incubated with the soluble enzyme preparation (5 ml; 47 mg protein; pH 8.0) for 2 hr at 10 C. The incubation mixtures were treated as described in the case of the incubation of the labeled 10-hydroxystearate. No conversion (<0.1%) of the labeled 5-, 6-, 7-, 8-, 11-, 14-, 15- and 16-hydroxystearates to monounsaturated fatty acids was observed.

Incubations of [1-^{14}C]-Stearolic Acid With Enzyme

[1-^{14}C]-Stearolic acid (1.5 x 10^{5} cpm) was dissolved in 0.02 N KOH (0.05 ml) and incubated with the soluble enzyme preparation (5 ml; 47.5 mg protein; pH 8.0) for 2 hr at 10 C. The incubation mixture was heated on a steam bath for 30 min with 10% methanolic KOH. After acidification to pH 1 with 2 N HCl, the mixture was extracted three times with ether (25 ml portions). The combined ether extracts were washed with water and dried over anhydrous sodium sulfate. Thin layer radiochromatographic analysis on a Silica Gel G plate (solvent: petroleum ether-ether-acetic acid, 60:40:1) along with carrier stearolic acid and 10-ketostearic acid indicated that less than 1% of the radioactivity had the chromatographic mobility of 10-ketostearic acid (Fig. 1). Since 9-ketostearic acid behaves similarly in this chromatographic system, the findings also indicate little, if any, formation of labeled 9-ketostearate.

DISCUSSION

The findings reported herein constitute an extension of our previous studies relative to the substrate specificity of the enzyme in question. A number of hydroxystearic acids were tested as possible substrates for the enzyme. Only 10-hydroxystearic acid served as a substrate. In this case, these and findings previously reported (7) are compatible with a stereospecific utilization of only the D (or R) isomer of 10-hydroxystearic acid. Stearolic acid does not serve as a significant substrate for the enzyme.

ACKNOWLEDGMENT

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Lipid Monolayers: Influence of Lipid Film and Urea on the Surface Activity of Staphylococcal α-Toxin[1]

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ABSTRACT

Under conditions in which neither ribonuclease nor lysozyme formed a film by either spreading or adsorption at the air-water interface, the surface activity of staphylococcal α-toxin and streptolysin S in the absence of lipid was greatly enhanced by the presence of 6 M urea in the hypophase. The influence of urea on the anchoring of the protein was quantitatively similar to that of a lecithin film. Admixture of up to 50 mole % cholesterol to egg lecithin preserved the lecithin character of the lipid monolayer in the penetration of α-toxin, suggesting probably binding of cholesterol to phosphatidyl choline in the presence of excess lecithin. Penetration of α-toxin and streptolysin S into the air-water interface was inhibited by lipid monolayers containing gangliosides. The inhibition, however, was removed when the subphase contained 6 M urea. The resulting penetration curve of α-toxin was identical with the penetration curve of the protein into the lecithin film in the absence or in the presence of urea. In general, the denaturing agent causes the protein to acquire a hydrated conformation, from which restructuring in the low dielectric constant of air or lipid is favored and brings about results comparable with those effected by the lipid. Pre-incubation of α-toxin with gangliosides or sulfatides caused a marked decrease in film penetration of the toxin into lecithin monolayers. Unlike α-toxin, streptolysin S was extremely sluggish in penetration of the air-water interface and of several lipid monolayers. Protein concentration, a complement of either lipid or membrane protein, and specific molecular organizations of membranes must be taken into account in all cases; they, in particular, may be the basis for the enhanced cytolytic activity of streptolysin S in vivo.

INTRODUCTION

The involvement of microbial exotoxins in the lysis of host cell membranes prompts interest in uncovering the surface properties and mechanisms which make these proteins so intensely membrane bound. Since the pioneer experiments of Doty and Schulman (1) and Eley and Hedge (2), lipid monolayers have been used to probe the ability of proteins to interact with membrane surfaces.

The approach of film penetration is one in which a lipid monolayer is made at a given initial pressure (π_i), protein is injected under the lipid monolayer, and the increase in film pressure ($\Delta\pi$) is measured as a function of time. Although rate and magnitude of film pressure increase are generally interpreted as a measure of interaction, the values of $\Delta\pi$ do not relate either to the quantity of protein present in the film nor to any lipid-protein complex, hydrophobic or hydrophilic. Recent studies have revealed some interesting aspects of the general process of film penetration (3-6). For example, the quantity of protein incorporated in the film at a given value of $\Delta\pi$ can be measured; it is now possible to identify mechanisms by which proteins reach into the hydrophobic regions of the lipid, anchor at the air-water or lipid-water interface, and bind on the hydrophylic groups of lipid or protein that extend into the aqueous phase.

By means of this approach information was obtained about the interaction of staphylococcal α-toxin with lipid monolayers (7). Unlike ordinary globular proteins as ribonuclease and lysozyme, α-toxin readily spread as a film; it formed unusually thick films. In the penetration of lipid monolayers, the toxin revealed a general dependence on the chemical structure of the lipid; as observed with other proteins, the $\Delta\pi$ values were in the order cholesterol > egg lecithin > sphingomyelin. Penetration of the toxin into lipid films was strongly inhibited by gangliosides. The observations at the air-water interface were deemed significant in

[1]This work is taken from the dissertation presented by A.R. Buckelew, Jr. in partial fulfillment of the requirements for the Ph.D. degree in the Department of Microbiology at the University of New Hampshire, 1968. The monolayer experiments were done at the Albert Einstein College of Medicine.

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relation to the ability of α-toxin to bind gangliosides and adsorb on and coat membrane surfaces.

Since little is known about the mechanisms by which proteins form films and since conformational changes have been invoked in the transport of proteins from solution into films (3), the attention should be directed towards investigating the surface activity of proteins under conditions which are known to modify protein conformations. The present study deals with the influence of urea and lipid medium on the penetration of staphylococcal α-toxin into the air-water interface. Another cytolytic protein, streptolysin S, was studied for comparison.

EXPERIMENTAL PROCEDURES

Materials

Isolation of purified α-toxin, from a human strain of *staphylococcus aureas* has been described (7); the preparations were biochemically and immunochemically homogeneous. The toxin migrated as a uniform band in gel filtration through Sephadex G-75, in acetate strip and agarose gel electrophoresis. In immunodiffusion, α-toxin gave only one precipitin line against Lederle's heterologous antistaphylococcus sera. The stock solution of purified toxin was stored in 80% saturated ammonium sulfate at 4 C. When needed for study, the protein was recovered in a pellet by centrifugation for 30 min at 24,000 x *g*. The preparation was dissolved in the cited phosphate buffer at a concentration of 5 mg/ml. The toxin's preparations were free of lipid. They did not contain lipid phosphorus, and no lipid was found by thin layer chromatography of the chloroform phase when abundant aqueous protein was shaken with chloroform-methanol 2:1.

Streptolysin S, 15,000 hemolytic units per milligram, was obtained from the late R. Rowen and was prepared by a known method (8).

Bovine ribonuclease, 2x crystallized, (Lot 6RLA), and lysozyme, a salt free preparation from egg white (Lot LYF-6JA), were purchased from Worthington Biochemical Corporation, Freehold, N.J. Protein solutions of 1 to 5 mg/ml in 0.04 M phosphate buffer containing 0.1 M NaCl, pH 7, were stored at 2 C for not longer than three days.

Urea, reagent grade, was recrystallized twice from 80% ethanol in the cold (10); salts were dissolved in freshly prepared solutions of 6 M urea in water. The urea solutions were used within 24 hr. Under these conditions, it was found (10), urea did not damage the proteins by the carbamylation reaction (11).

Lipids. Egg lecithin and cholesterol, products of Silvana Chemical Co., Millburn, N.J. were homogeneous by thin layer chromatography; a preparation of "highly purified mixed gangliosides from beef brain gray matter" was obtained from Rapport and consisted primarily of disialogangliosides (7). The lipid solutions, 0.5 mg/ml for monolayer experiments were prepared fresh in 85:15 chloroform-methanol and stored at 2 C for not longer than three days. The organic solvents, reagent grade, were redistilled before use.

Incubation of α-Toxin With Lipids. After removal of the organic solvent under nitrogen at room temperature, the lipid was dispersed in buffer containing an equal weight of α-toxin. The final concentrations were 5 μg/μl of each, lipid and protein. The mixture was left to stand with occasional gentle mixing for ½ hr at 23 C; an aliquot containing the desired quantity of α-toxin was injected under the lipid monolayers in the usual way.

Apparatus and Procedure. Surface pressure was determined as $\pi = \gamma_o - \gamma$ after measurement of surface tension of the water phase without film (γ_o) and with the film (γ). For the purpose, a sandblasted platinum blade of 5.03 cm perimeter was suspended from a torsion balance. Surface potential was measured by a radioactive ($^{226}R_a$) air electrode. The techniques have been described (3,4,13). Purification of the water was also described (7).

The trough consisted of a crystallizing dish partitioned into a large film area (18 cm^2) and a small service area. Barrier and inside surface of dish were coated with paraffin. Blade and radioactive electrode were positioned over the large area. The hypophase, 50 ml, was mixed gently with a magnetic bar.

The surface activity of the protein was determined as its ability to form a film by spreading or adsorption and to penetrate lipid monolayers. Protein solution of 1 to 5 mg/ml in a Hamilton microsyringe was either applied on the surface or injected into the hypophase. Details of these techniques and a micro-Lowry method for the determination of protein in the hypophase have been described (3).

RESULTS

Neither ribonuclease nor lysozyme made a film by spreading on phosphate buffer either in the absence or in the presence of 6 M urea. In contrast, rate and extent of pressure increase by α-toxin on 6 M urea was markedly greater than on phosphate buffer alone (Fig. 1); strikingly, the saturation pressure on urea is much higher than that in the absence of urea.

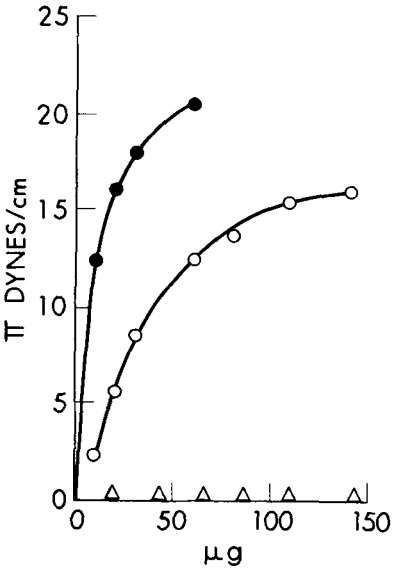

FIG. 1. Influence of 6 M urea in subphase on spreading ability of α-toxin -●-, as opposed to α-toxin in the absence of urea -○- and ribonuclease on 6 M urea -△-. Urea solution was first prepared in water, then the phosphate and NaCl salts were dissolved. Hypophase: 0.04 M phosphate buffer, in 0.1 M NaCl, pH 7.0, 25 C, with or without urea. Protein was applied on the surface (3).

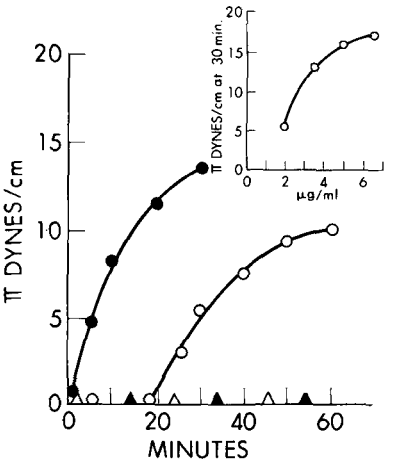

FIG. 2. Formation of film by adsorption of protein from subsolution. -△- ribonuclease; -▲-, streptolysin S; -○-, α-toxin; -●-, rat HDL-protein. Hypophase: 0.04 M phosphate buffer in 0.1 M NaCl, pH 7.0, 25 C Protein, 2 μg/ml, was injected in the hypophase (3). Inset: Effect of concentration of α-toxin on its film formation (π).

Under conditions in which neither ribonuclease, lysozyme, nor streptolysin S formed a film by adsorption, α-toxin surfaced rapidly after a lag period of 17 min (Fig. 2). As in film spreading (7), in the ability to surface from solution, toxin was between the apoprotein of high density lipoprotein and ribonuclease. A slight difference in the surface denaturation of the apolipoprotein studied by Camejo et al. (9) and the one that we used could be due to some difference in the ages of preparations. Surface properties of proteins can change appreciably from preparation to preparation and with aging of the protein solution (7). The inset of Figure 2 shows a smooth (exponential) relationship between film pressure (surface activity) and toxin concentration in phosphate buffer; a saturation pressure of 16 dynes/cm was attained in 30 min with a protein concentration of 7.5 μg/ml. The same saturation pressure was obtained by spreading (Fig. 1).

A marked influence of 6 M urea was seen also on the ability of toxin to adsorb at the interface (Fig. 3). The toxin had a lag period of 17 min in phosphate buffer, whereas the pressure rise was instantaneous and continuous in the presence of urea. Still more remarkable was the effect of urea on streptolysin S, which did not make a film from phosphate buffer but did so, readily, from 6 M urea, though not as rapidly as α-toxin did.

The toxin showed penetration of the uncovered air-water interface after a lag period of 17 min; in contrast it penetrated monolayers of egg lecithin readily (Fig. 4). In the presence of 6 M urea, rate and extent of penetration of α-toxin into egg lecithin films were slightly greater than in the absence of urea. When galactose, glucose or sucrose 0.1 M was present in the hypophase, the penetration curve of toxin into egg lecithin was identical with that in the absence of the sugars.

Rate and extent of penetration of toxin into ganglioside films were remarkably smaller than those with phosphatidyl choline (Fig. 5). Introduction of urea in the subphase, however, caused the values of $\Delta\pi$ to reach those obtained with lecithin (PC) and nearly the same values obtained with the adsorption of toxin in the absence of lipid but in the presence of 6 M urea; film penetration ($\Delta\pi$) in both cases (namely with or without lipid) shows a marked and similar dependence on the concentration of urea (Fig. 5 inset).

Streptolysin S, which did not build a film pressure in the absence of lipid, penetrated lecithin films at a much smaller rate than α-toxin did; and failure of Streptolysin S to penetrate ganglioside was conspicuous (Fig. 6). However, high concentration of urea caused a greatly enhanced pressure increase also with streptolysin.

When α-toxin was incubated with equal

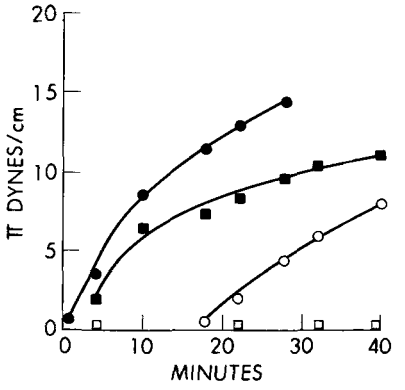

FIG. 3. Influence of urea on the ability of α-toxin to form a film by adsorption of protein from hypophase. -○-, α-toxin in the absence of urea; -●-, α-toxin with 6 M urea in hypophase; -□-, streptolysin S; -■-, streptolysin S with 6 M urea in hypophase. Hypophase: 0.04 M phosphate buffer in 0.1 M NaCl, pH 7.0, 25 C, with or without urea. Protein, 2 μg/ml, was injected in the hypophase.

weights of ganglioside or sulfatide prior to injection under lecithin monolayers, penetration was depressed markedly (Fig. 7). No change, however, was observed in the penetration curve of lecithin when α-toxin was preincubated with lecithin or sphingomyelin.

As with all the proteins investigated, penetration of α-toxin was largest with cholesterol films. However, mixed films containing 60 mole % or more phosphatidyl choline in cholesterol exhibited the same penetration curve of lecithin (Fig. 8).

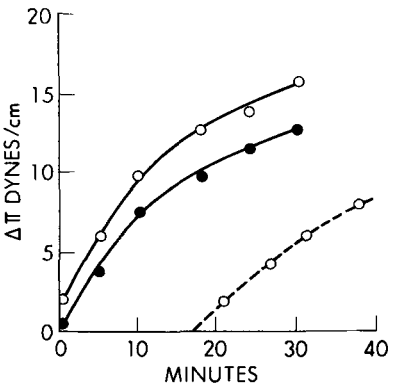

FIG. 4. Influence of lipid film and urea on the adsorption of α-toxin from hypophase. --○--, α-toxin in the absence of urea; -●-, penetration of α-toxin in the absence of urea into a monolayer of phosphatidyl choline (egg lecithin); -○-, penetration of α-toxin from hypophase containing 6 M urea into lecithin monolayer. Initial pressure of lipid film, 2 dynes/cm. Hypophase: 0.04 M phosphate buffer in 0.01 M NaCl, pH 7.0, 25 C, with or without urea. Protein, 2 μg/ml, was injected in the hypophase.

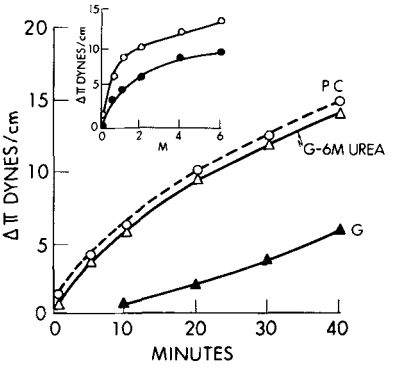

FIG. 5. Penetration of α-toxin into monolayers of gangliosides (G). -▲-, ganglioside on phosphate buffer; -△-, ganglioside on phosphate buffer containing 6 M urea; by comparison, --○--, egg lecithin (PC) on phosphate buffer. Inset: Effect of concentration of urea (M) in hypophase on the penetration of α-toxin into ganglioside monolayers. -●-, at 10 min; -○-, at 20 min. Lipid film at πi = 2 dynes/cm. Hypophase: 0.04 M phosphate buffer in 0.1 M NaCl, pH 7.0, 25 C, with or without urea. Protein, 2 μg/ml, was injected in the hypophase.

DISCUSSION

Studies of surface activity of proteins are aimed at elucidating the mechanisms and conditions by which proteins interact with membrane surfaces, lipid or protein. A detailed analysis of lipid-protein interactions in monolayers can develop from a consideration of simultaneous measurements of surface tension, surface potential and surface viscosity (6). However, the state of the experimentation and interpretation of the electrical and rheological properties of lipid, protein and lipid-protein systems at the air-water interface does not

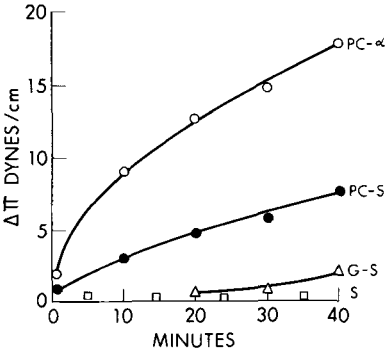

FIG. 6. Adsorption of streptolysin S at the air-water interface. -□-, In the absence of a lipid film; -△-, ganglioside (G) films; -●-, egg lecithin (PC) films; -○-, by comparison, adsorption of α-toxin into lecithin film. Initial pressure of lipid film, 2 dynes/cm. Hypophase: 0.04 M phosphate buffer in 0.1 M NaCl, pH 7.0, 25 C. Protein, 2 μg/ml, was injected in the hypophase.

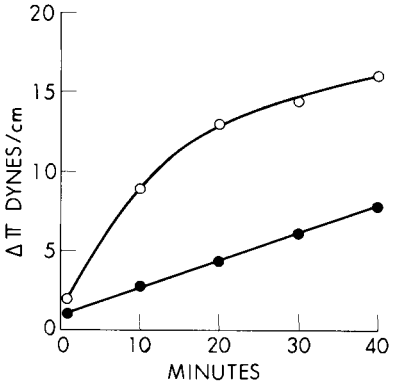

FIG. 7. Influence of incubation of α-toxin with ganglioside on the penetration ($\Delta\pi$) of lecithin monolayers. -●-, After incubation; -○-, without incubation (see Methods). Initial film pressure 2 dynes/cm. Hypophase: 0.04 M phosphate buffer in 0.1 M NaCl, pH 7.0, 25 C. Protein, 2 μg/ml, was injected alone or with ganglioside (incubation mixture) in the hypophase.

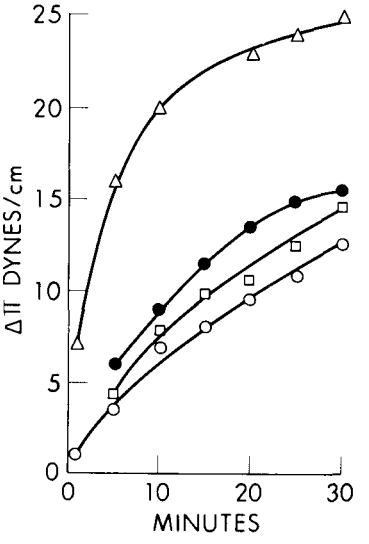

FIG. 8. Effect of lecithin on the penetration ($\Delta\pi$) of cholesterol monolayers by α-toxin. -△-, Cholesterol; -□-, cholesterol-lecithin 40:60 molar ratio; -●-, cholesterol-lecithin 50:50; -○-, lecithin film. Initial film pressure 2 dynes/cm. Hypophase: 0.04 M phosphate buffer in 0.1 M NaCl, pH 7.0, 25 C. Protein, 2 μg/ml, was injected in the hypophase.

warrant a satisfactory description of any such system at present. Most of the discussion will therefore center on the surface tension data.

Although the values of $\Delta\pi$ following injection of protein at the air-water interface relate rigorously to the surface valence of the protein and thus to the number of its polar groups that anchor at the interface, the only way to tell about mechanisms of protein penetration and lipid-protein interaction is from a concerted analysis of the kinetic ($\Delta\pi$-time) curves and the quantities of protein effectively present in the film. The study, however, is not complete unless one probes the lipid and protein surfaces with ions, lypolytic and proteolytic enzymes, to determine the relative position of ionized groups, lipid and protein in the membrane topography (4). Although, unfortunately none of all that was done with α-toxin, the surface tension data provide poignant messages concerning the transport of protein from bulk to film. This information then becomes the basis for the further exploration that will be performed when the physical meaning of both surface potential and surface viscosity will be better understood.

As we stated elsewhere (7), we chose not to use nor to interpret "equilibrium" or saturation data. The complexity of the mechanistic and thermodynamic processes attending the transport of protein from water to film (3) is beyond human comprehension at present. The wisdom of the approach is in the selection of simple parameters that permit a meaningful comparison of the available systems; rates and extent of π increase in the early phase of the interaction, a few seconds or minutes, are more relevant to biology than the dogma of an equilibrium state which may not even exist and about which we understand literally nothing. We know that one can have considerably different quantities of protein in the film for the same value of π or $\Delta\pi$ (3), and the quantities of protein calculated by use of equilibrium thermodynamics (9) can differ by one or two orders of magnitude from the quantities of proteins actually found in the film (4). Rather, we may ascertain first the molecular meaning of surface tension, surface potential, surface viscosity, structure and composition of the membrane components. Therefore, until that is done, any attempt to use or calculate thermodynamic or kinetic parameters past the simple rate expression $d\pi/dt$ is bound to be a vain intellectual exercise fraught with presumption, confusion and frustration (4).

Influence of Lipid Surfaces

In the philosophy of the architecture of membrane surfaces, phosphatidyl choline is the lipid which supposedly does not bind subphase protein, can be compressed sufficiently from low film pressures and, in the process, can provide new areas for appreciable quantities of protein to be incorporated in the membrane (3,4,6). By this mechanism, referred to as free penetration, the protein anchors at the interface independently of the lipid, although the

latter's presence influences the results markedly; lecithin provides a medium of low dielectric constant which favors structuring of the protein in the film. As several other proteins do, α-toxin enjoys this mechanism with particular alacrity. The most remarkable phenomenon is shown in Figure 4. At a concentration of 2 μg/ml in the subphase, toxin does not build a film for 17 min, but it does instantaneously when the surface is covered with lecithin at an initial film pressure of 2 dynes/cm. In the absence of lipid surfaces, many proteins, including the apoprotein of human high density lipoprotein (3), show a considerable lag period in the adsorption from the subphase.

Such lag period vanishes either when the surface is covered with a lipid or protein film of at least 2 dynes/cm pressure, or when the protein concentration in the subphase is sufficiently high, i.e., 10 μg/ml. This requirement varies from protein to protein. The nature of the interactions leading to the formation of the protein film can be only speculated at present (3).

Although in making a film by spreading (Fig. 1), α-toxin is slightly less surface active than mitochondrial structural protein and markedly less surface active than the apoprotein of high density serum lipoproteins, α-toxin is as active as the latter and much more active than the mitochondrial structural protein in forming a film by adsorption from dilute solutions. In contrast, streptolysin S, which also interacts with various types of biological membranes (14), displayed much less surface activity than α-toxin did. This may mean that the requirements of streptolysin S could be higher protein concentrations in the subphase [as it is the case with mitochondrial structural protein (3)], and/or a specific membrane surface that stimulates surface activity in the cytolytic protein. Such a situation could be related to a mechanism of binding-mediated penetration (4), operated by relatively large contents of cholesterol, glycolipids, phosphatidyl serine and phosphatidyl ethanolamine in the membrane. The hydrophilic groups of these lipids are assumed to stimulate the protein's surface activity and pull proteins into lipid membranes with special intensity (3,4,6).

Inasmuch as activation of surface activity of the protein may be provided by the lipid, it can be appreciated that globular proteins, ribonuclease and lysozyme, which do not form a film by spreading on water or dilute salt solutions, not even on 6 M urea, are adsorbed when spread on a lipid-covered surface such as a low pressure film of phosphatidyl choline (lecithin). This means that the lipid provides a surface against which these proteins expand, and a medium of low dielectric constant in which the uncoiled protein then anchors and restructures (6).

Influence of Urea

Instructive in that respect are the kinetic curves for the adsorption of toxin in Figures 4 and 5. In experiments on the adsorption from the subphase, α-toxin showed a lag period of 17 min, after which the rise in film pressure was nearly linear over the remaining time; the observation lasted 40 min. Under the same conditions, streptolysin S did not build any pressure for 60 min (Fig. 2, 3). However, both streptolysin S and α-toxin showed a remarkable film formation ability when urea 6 M was present in the subphase (Fig. 3). Interestingly, the curve of penetration of α-toxin into a lecithin monolayer (Fig. 4) is very similar to the curve of film formation by toxin in 6 M urea (Fig. 3). Rate and extent of penetration of toxin in lecithin films were increased but only slightly by urea (Fig. 4). This may indicate that, although the mechanisms may be different, the lipid surface activates the protein as much as a large urea concentration does. It should be also noted that in the absence of both urea and lipid, once the pressure begins to build at 17 min, the rate of pressure increase with toxin alone resembles the rate of penetration of toxin into the lecithin monolayers (Fig. 4). Although stimulation of the surface activity of toxin by urea and by the lipid surface could be quite different processes, the similarities of the effects cannot be overlooked. The results show that once a film of moderate pressure of either lipid or protein is formed, it assists the further incorporation of protein.

Structural Requirements of Membrane Bound Proteins

The increase in surface activity exhibited by α-toxin in the presence of increasing concentrations of urea implies that unfolded protein chains are more surface active than native globular structures. However, from a qualitative standpoint, it is also clear that since most of the effect is obtained with 2 M urea (Fig. 5, inset) and since much greater concentrations of urea are needed for full denaturation (15), complete unfolding of the protein may not be necessary for manifestation of surface activity. An expanded protein structure, say with 2 M urea, may be sufficient activation for the protein to anchor and restructure at the interface.

Since formation of thick films by the toxin could express a mechanism of aggregation of

the toxin near the interface, the greater surface activity observed in the presence of urea could bear some similarity to a process of disaggregation of α-toxin complexes (16). Smaller particles and linear structures could account for an easier access of protein moieties to the interface.

The absence of disulfide bridges in α-toxin (5,17) could be an important structural feature to account for its surface activity and its sensitivity to urea. Since disulfide bridges are not found in membrane proteins and in lipoproteins as well (7,18), the surface activity of HDL-protein was attributed in part to the flexibility of the polypeptide chain caused by the absence of disulfide bridges in that molecule (9). This is not to say, however, that proteins containing disulfide bridges are not sensitive to urea, in fact, serum albumin and γ-globulin were very sensitive (albumin>globulin, unpublished results). The influence of urea on the adsorption of ribonuclease and lysozyme is not marked, probably because the globular structures of these proteins are not very sensitive to urea (19). Yet these two proteins penetrate lipid monolayers as readily as α-toxin. Similarities are as striking as differences. However, although mechanisms and kinetics by which urea and lipid elicit surface activity of the protein could be different, the extent of the $\Delta\pi$ effect is the same.

Relation of π or $\Delta\pi$ Values to Structure of Film Protein

Remarkably, when α-toxin was spread on 6 M urea the saturation pressure was far above 20 dynes/cm as opposed to 16 dynes/cm in the absence of urea. Although protein films contain large quantities of water as compared to films of saturated lipids at a pressure of 40 to 50 dynes/cm, the hydrophobic moieties of the anchoring structures of protein must extend 15 to 25 A well above the π-interface to obtain the stabilities observed with protein films at pressure of 15 to 25 dynes/cm. The film structures therefore cannot be those of an extended polypeptide chain, 5 to 7 A in thickness; they are more likely clusters of parallel vertical or horizontal coils (6). In films, as α-toxin's containing large quantities of protein, the hydrophobic π-moieties cannot be as sheets of β-keratin structures. These would be very viscous and have very low, if any, measurable surface pressure, and very low surface potentials (20), up to 100 mv at the most. The protein films in question have high surface potentials 200 to 300 mv and are not so viscous.

The foregoing observations argue in favor of recent evidences whereby film proteins have globular α-helix or random coil and not the β-structures of extended denatured polypeptide chains (21,22). The fact that denaturation of protein by urea favors transport of protein into the film is explained by a mechanism in which hydrated, expanded or extended, protein structures are precursors of hydrophobic and bimodal protein. Folding of such hydrophilic structures into hydrophobic compact structures proceeds with release of water and free energy (3,6,19). Such processes are greatly favored by the low dielectric constant of the air, lipid or protein structures already anchored at the interface. Membrane proteins differ from ordinary nonstructural proteins in that the former possess a bimodal or amphipathic behavior in virtue of a structural asymmetry that makes the membrane proteins or the membrane bound peptides (23) as surface active as the lipid.

In the light of all these considerations, a prediction is in order. Namely, when urea is effective in raising π or $\Delta\pi$ values, the hydrophobic part of the bimodal film protein (π forms) above the π interface will have more α-helix than the aqueous globular protein. In contrast, the hydrophilic omega protein forms, responsible for surface viscosity below the π interface (6), will contain keratin-like β-structures, Surface potential, surface viscosity and infrared spectroscopy studies now in progress are a test of that prediction (3,4,6).

Interaction of α-Toxin With Ganglioside Monolayers

A truly remarkable feature of α-toxin and streptolysin S was their failure to penetrate ganglioside films (Fig. 5 and 6). Inhibition of penetration of lecithin films was proportional to the ganglioside content and was already appreciable with 10 mole % of the glycosphingolipid. This behavior of α-toxin has some biological significance, since preincubation of toxin with gangliosides reduces the lethal effects of toxin (24). In vitro, preincubation of α-toxin with gangliosides depressed considerably the penetration of toxin into lecithin monolayers (Fig. 7). Sulfatide behaved similarly. Preincubation of the toxin with lecithin or sphingomyelin had no effect.

Binding of the cationic α-toxin with the hydrophilic groups of the acidic glycosphingolipids below the π-interface of the lipid film and inhibition of $\Delta\pi$ mechanisms (3,4) can explain the results. These aspects of the surface behavior of α-toxin and gangliosides bear a relation to the binding of tetanus toxin to brain membranes and to the role of gangliosides in

preventing the binding and the suppression of electrical activity of brain by the toxin (25,26).

The presence of 6 M urea in the subphase abolished the inhibition of toxin penetration by gangliosides (Fig. 5). Although one cannot exclude that urea can act also on the ganglioside, it is clear that a toxin unfolded by urea implants interfacial structures much more rapidly than it can be bound on the gangliosides, irrespective of whether this binding takes place or not in the presence of urea. It should be noted that rate and extent of film penetration ($\Delta\pi$) of toxin from 6 M urea into a ganglioside film were the same as in the absence of lipid and as the penetration of lecithin films in the absence of urea. The influence of the concentration of urea on the penetration of α-toxin into the air-water interface (Fig. 5, inset) was the same whether the surface was covered with ganglioside (at 2 dynes/cm pressure) or had no lipid.

Interaction of α-Toxin with Lecithin-Cholesterol Films

Monolayers of phosphatidyl choline (egg lecithin) containing up to 40 mole % cholesterol preserved a lecithin character with regard to penetration by α-toxin (Fig. 8). This observation parallels previous findings with a cholesterol-lecithin-globulin system (27). The meaning is that a mechanism of free penetration, which operates when excess lecithin is in the film, is replaced by or coupled with a mechanism of binding-mediated penetration, typical of cholesterol, as soon as free cholesterol becomes available to the protein; subsequently, penetration ($\Delta\pi$) increases linearly with the cholesterol concentration. The lipid composition at which the transition occurs is affected by concentration of protein and rate of mixing of the subphase (6).

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Comparative Citrus Fatty Acid Profiles of Triglycerides, Monogalactosyl Diglycerides, Steryl Esters and Esterified Steryl Glucosides

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ABSTRACT

Vesicular lipids from six orange and two tangor varieties were extracted, purified and separated by chromatography into triglycerides, monogalactosyl diglycerides, steryl esters and esterified steryl glucosides. Methyl esters of the fatty acids found in these four lipids were prepared and analyzed by gas liquid chromatography. Each of the eight citrus varieties gave a series of four profiles which could be distinguished from the others. The Temple tangor has four profiles all showing a large percentage of linolenic acid. In all varieties steryl esters and to a lesser extent esterified steryl glucosides contain relatively large concentrations of 22:0 to 26:0 saturated acids. The profiles differ markedly from the patterns found for these four lipids in other higher plants studied.

INTRODUCTION

The most important commercial fruit produced by the United States belong to the genus *Citrus*. Considerable investigations have been conducted on the botanical and horticultural science of *Citrus* (1); however there exists limited information on citrus chemotaxonomy. The most comprehensive chemotaxonomic studies have been conducted by Scora et al. (2) on essential oils, Albach and Redman (3) on flavanones, and Nordby and Nagy (4,5) on fatty acid profiles of citrus juice and seeds.

Fatty acid profiles of plant tissue have generally been determined on the total extractable lipid. This method, while informative, cannot always differentiate minor differences in fatty acid patterns of closely related species (5). Differences in patterns of specific constituents of various classes of lipid from citrus fruit vesicles may correlate with taxonomic classification. To test this premise and further our knowledge of citrus chemotaxonomy, fatty acid profiles were determined for four lipid classes: triglyceride, monogalactosyl diglyceride (MGDG), steryl ester and esterified steryl glucoside (ESG). These four lipid classes were studied in six orange varieties (1) of *Citrus sinensis* Osbeck, namely Walker Early, Parson Brown, Hamlin, Washington Navel, Pineapple and Valencia, and two tangors, viz. Temple (*C. sinensis* x *C. reticulata*) and Temple x Kinnow [(*C. sinensis* x *C. reticulata*) x *C. reticulata*].

MATERIALS AND METHODS

Isolation of Vesicular Lipids

Valencia, Hamlin, Parson Brown and Walker Early oranges were obtained from local groves. Pineapple and Washington Navel oranges and the Temple x Kinnow tangor were obtained from Whitmore Experimental Farm, (Crops Research Division, USDA, Orlando, Fla). The Temple tangor was obtained from a local market. All samples were collected at the time of their respective peak maturities. Fruits of the eight varieties were cut in half and the intact juice sacs (vesicles) were carefully separated from core, peel, seeds and carpellary membrane with the aid of a citrus spoon. The samples were freeze-dried to a powder possessing a moisture content no greater than 4% and stored at 5 C until lipid extractions were carried out. Lipids were extracted and purified from 15 g of juice sac powder by the method previously described for orange juice powders (6). Each variety sample was run in triplicate.

Standards

Fatty acid methyl esters were obtained from the Hormel Institute, Austin, Minn. A polar lipid standard containing monogalactosyl diglyceride, lecithin and cerebroside was obtained from Applied Science Laboratories, State College, Pa.

Column Chromatography

The total purified lipid (ca. 150 to 200 mg) was dissolved in absolute $CHCl_3$ (no ethanol stabilizer) and percolated onto an 0.9 x 30 cm column containing 9 g Merck Ag, 70 to 325 mesh silica gel (Brinkmann Instruments, Westbury, N.Y.). Neutral lipids were eluted with

[1]So. Market. Nutr. Res. Div., ARS, USDA.

150 ml absolute $CHCl_3$, glycolipids with 200 ml $CHCl_3$-MeOH (95:5) and the remaining polar lipids with 200 ml MeOH. The MeOH fraction was not subjected to any further examination in this study. Column aliquots (25 ml) were monitored by thin layer chromatography (TLC) to insure elution completeness for each group of lipids.

TLC and Detection Reagents

Preparatory separation of the neutral lipid and glycolipid fractions was accomplished on precoated Silica Gel G plates (20 x 20 cm, 250 μ, Analtech, Inc., Wilmington, Del.). These nonactivated plates were developed at room temperature in chambers lined with filter paper. The following solvent systems were used: hexane-ethyl ether (90:10) containing 0.1% di-tert-butyl-cresol (BHT) for separation of triglycerides and steryl esters from other neutral lipids and $CHCl_3$-MeOH (85:15) for separation of esterified steryl glucosides and monogalactosyl diglycerides from other components in the glycolipid fraction, namely steryl glucoside, cerebroside and resin acids. Two-dimensional TLC on Silica Gel H in solvent systems previously reported (6) was used to confirm the absence of overlap of lipids in the glycolipid fraction. Other solvent systems employed were: benzene-hexane (140:80) for purification of methyl esters from triglyceride and ESG hydrolysis; $CHCl_3$ for separation of products from steryl ester hydrolysis (free sterols and free fatty acids); and 2-propanol-ethyl acetate-water (3:2:1) for qualitative analyses of products derived from transmethylation of ESG and MGDG. For nonselective detection, spots were made visible on both G and H plates by staining with iodine vapor, phosphomolybdic acid (10% in absolute ethanol), or charring (7). For detection of glycolipids and methyl glycosides, anisaldehyde-H_2SO_4 and α-naphthol-H_2SO_4 (8) were employed; and for sterol-containing lipids, spraying with 50% sulfuric acid followed by heating at 140 C for 10 min produced distinct colors. Lipids separated by one-dimensional preparative TLC were detected by spraying with Rhodamine 6G and viewed under UV light.

Preparation of Fatty Acid Methyl Esters

Triglycerides, separated by preparative TLC and visualized with Rhodamine 6G, were scraped from the plate as a band and eluted with ethyl ether. Methyl esters were prepared by the transesterification BF_3-MeOH method previously described (4). The esters were further purified by TLC. The MGDG band was scraped from the plate and transesterified directly with BF_3-MeOH. No prior removal of MGDG from the silica gel by elution was conducted. Steryl esters were eluted from silica gel with ethyl ether, concentrated to dryness under nitrogen and hydrolyzed with 3 ml 6% KOH in 95% ethanol in 10 ml sealed acetylation tubes (Regis Chemical Co., Chicago) for 1 hr. After neutralization the products were extracted into cyclohexane and chromatographed on TLC plates. The free fatty acid band was scraped from the plate and methylated directly with BF_3-MeOH. Esterified steryl glucosides were eluted from silica gel with $CHCl_3$-MeOH (1:1), concentrated to dryness and transmethylated (9) by reacting the sample in sealed 10 ml acetylation tubes with 4 ml 0.5 *N* anhydrous HCl in MeOH containing 0.1% BHT for 22 hr at 75 C. Upon removal of solvent and catalyst (in vacuo, under nitrogen at 30 C) the products were chromatographed on TLC plates and recovered. For comparative purposes the ESG band of two samples was scraped from the TLC plates, transesterified directly in the presence of silica gel with BF_3-MeOH and the methyl esters purified by TLC. Representative methyl ester samples were hydrogenated (5) for confirmatory studies on equivalent chain lengths of the various acids.

Degradation Studies

Representative samples of ESG and MGDG were subjected to transmethylation under the above described conditions for methyl ester preparation. The products were separated by TLC and recovered. In like manner the products from hydrolysis of these two samples with 6% KOH in 95% ethanol were recovered and separated. A portion of the products was analyzed by TLC. The methyl glycosides were converted to their trimethylsilyl derivatives, extracted into hexane and dried according to the procedure of Mangold (10).

Gas Liquid Chromatography

Trimethylsilylated derivatives of the methyl glycosides were chromatographed isothermally on two separate 6 ft x 1/8 in. stainless steel columns. The first column packed with 10% UCW 98 on 100-120 mesh Chromosorb W was operated at 190 C with injection and detector temperatures of 235 C and a helium flow of 92 ml/min. The second column packed with 5% OV-1 on 100-120 mesh Gas Chrom Q (Applied Science) was operated at 160 C, with injector and detector at 235 C and helium flow of 60 ml/min. All retention times were related to the retention time of the TMS derivative of α-methyl glucose (11). Fatty acid methyl esters were analyzed on an 8 ft x 1/4 in. glass column

TABLE I

Fatty Acids of Triglycerides From Orange and Tangor Juice Sacs, %

Carbon No.	Walker Early	Parson Brown	Hamlin	Washington Navel	Pineapple	Valencia	Temple	Temple x Kinnow
14	0.96 ± .10	0.49 ± .06	1.04 ± .10	0.70 ± .10	1.19 ± .19	0.46 ± .05	0.77 ± .09	1.03 ± .09
15	0.23 ± .05	0.12 ± .03	0.27 ± .05	0.23 ± .05	0.16 ± .02	0.15 ± .03	0.28 ± .04	0.25 ± .09
16	9.97 ± .53	9.80 ± .46	10.51 ± .41	9.45 ± .41	9.90 ± .30	7.67 ± .22	8.21 ± .34	7.99 ± .18
16:1[b]	9.78 ± .60	9.30 ± .30	7.04 ± .29	10.41 ± .25	8.99 ± .28	7.79 ± .18	8.53 ± .44	8.58 ± .31
16:2 + I 18[c]	0.71 ± .13	0.90 ± .12	0.63 ± .14	0.73 ± .07	0.85 ± .15	0.81 ± .10	0.60 ± .18	0.53 ± .12
18	1.15 ± .13	1.36 ± .23	1.41 ± .22	1.21 ± .18	1.54 ± .21	0.83 ± .13	1.39 ± .09	1.47 ± .13
18:1	37.91 ± .58	41.07 ± .33	33.22 ± .38	42.35 ± .34	36.89 ± .43	29.90 ± .48	32.24 ± .45	37.40 ± .72
18:2	27.74 ± .64	25.74 ± .43	28.93 ± .61	22.02 ± .47	28.01 ± .45	35.54 ± .43	13.02 ± .32	31.45 ± .64
18:3	10.78 ± .40	10.06 ± .15	16.44 ± .23	11.72 ± .67	11.56 ± .37	16.16 ± .20	34.19 ± .53	10.73 ± .30
20:1	0.77 ± .12	1.16 ± .14	0.51 ± .09	1.18 ± .12	0.91 ± .17	0.69 ± .13	0.77 ± .15	0.57 ± .18

[a]Mean ± SD of nine determinations.

[b]Number of double bonds.

[c]Unresolved peak of 16:2 and iso 18.

TABLE II

Fatty Acids of Monogalactosyl Diglycerides From Orange and Tangor Juice Sacs, %

Carbon No.	Walker Early[a]	Parson Brown	Hamlin	Washington Navel	Pineapple	Valencia	Temple	Temple x Kinnow
12	Trace[b]	0.11 ± .01	Trace	Trace	Trace	0.27 ± .07	0.32 ± .07	0.37 ± .02
14	0.26 ± .06	0.21 ± .01	0.25 ± .02	0.16 ± .03	0.16 ± .02	0.63 ± .13	0.63 ± .02	0.57 ± .02
15	Trace	Trace	Trace	0.18 ± .02	Trace	0.18 ± .04	0.39 ± .01	0.10 ± .02
16	4.81 ± .09	4.17 ± .11	5.26 ± .51	3.71 ± .50	3.52 ± .06	6.69 ± .18	6.36 ± .11	4.46 ± .69
16:1[c]	10.49 ± .50	10.59 ± .23	6.49 ± .22	10.94 ± .56	9.43 ± .22	6.14 ± .33	7.54 ± .16	6.43 ± .19
16:2 + I 18[d]	0.62 ± .05	0.63 ± .09	0.46 ± .05	0.54 ± .07	0.63 ± .06	0.48 ± .05	0.50 ± .05	0.48 ± .04
18	0.79 ± .10	0.52 ± .08	0.75 ± .06	0.96 ± .07	0.57 ± .05	0.60 ± .06	1.33 ± .05	0.91 ± .04
18:1	30.36 ± .88	30.71 ± .77	27.11 ± .27	28.86 ± .28	28.39 ± .55	27.06 ± .17	31.02 ± .25	24.06 ± .51
18:2	14.17 ± .30	18.14 ± .36	19.07 ± .50	13.88 ± .57	17.09 ± .16	26.44 ± .62	6.86 ± .15	14.76 ± .25
18:3	36.82 ± 1.13	33.36 ± .68	40.02 ± .73	39.44 ± .53	38.85 ± .29	31.21 ± 1.15	44.61 ± .34	47.10 ± .33
20:1	1.68 ± .12	1.56 ± .05	0.59 ± .06	1.33 ± .14	1.36 ± .17	0.30 ± .06	0.44 ± .07	0.76 ± .05

[a]Mean ±SD of nine determinations.

[b]Trace, less than 0.1%.

[c]Number of double bonds.

[d]Unresolved peak of 16:2 and iso 18.

containing 10% stabilized DEGS (Analabs, Inc., Handem, Conn.) coated on DMCS-treated Chromosorb W (100/120 mesh) utilizing on-column injection. Injection port and detector were at 230 C and the helium flow was set at 80 ml/min. The analyses were conducted isothermally at 190 C until the 20:1 ester had emerged. The temperature was then raised to 230 C at 60 C/min until the 22:0 to 26:0 methyl esters had emerged. Peak areas were measured with the aid of a disc integrator. Each ester sample was run in triplicate. Percentage compositions were calculated for those acids which were greater than 0.1% for two or more citrus varieties. For confirmational studies of equivalent chain lengths, samples of both natural and hydrogenated citrus esters were chromatographed along with standard esters on a DEGS column under programmed conditions of 2 C/min from 150 to 230 C and held at 230 C (4). All columns were run in an F & M Model 5750 gas chromatograph equipped with flame ionization detectors.

RESULTS AND DISCUSSION

The lipid extracts of juice sacs from oranges and tangors contain four major glycolipids: esterified steryl glucoside, steryl glucoside, monogalactosyl diglyceride and cerebroside, and two minor glycolipids: digalactosyl diglyceride and a sulfolipid. The four major glycolipids are exclusively eluted from a silica gel column with $CHCl_3$-MeOH (95:5) while the two minor glycolipids are found in the MeOH fraction. Analysis of the $CHCl_3$-MeOH column fraction by TLC reveals the following migration sequence (increasing R_f values): cerebrosides, steryl glucoside, MGDG, ESG, resin acids and, occasionally, traces of free sterol.

Esterified Steryl Glucoside

This class of lipids had R_f values on one- and two-dimensional TLC comparable to those reported for ESG from other plant sources (12-14). They stained positively for sugar and sterols and negatively toward ninhydrin and phosphate reagents. Upon methanolysis under acidic conditions free sterols, methyl glucoside and fatty acid methyl esters were the major products detected by TLC and GLC. Upon basic hydrolyses, steryl glucosides and free fatty acids were the products, thus confirming the sterol-glucose glycosidic linkage. This glycosidic linkage has been shown (13) to be quite resistant to alkaline hydrolysis.

Monogalactosyl Diglyceride

These glycolipids had the same chromatographic R_f values as that of standard MGDG and that reported in the literature (12,14,15). Color tests were positive for sugars but negative for sterols, free amino groups and phosphate. Upon methanolysis, methyl galactoside was detected by TLC and GLC analyses. Methyl esters were the only other product detected since glycerol was not determined.

Fatty Acid Composition

Table I shows the fatty acid composition of triglycerides found in the six orange and two tangor varieties. The five major acids, palmitic, palmitoleic, oleic, linoleic and linolenic, comprise 96% to 97% of all acids. Generally these five acids are in the approximate ratio 1:1:4:3:1, respectively. Two varieties of orange and one tangor, however, differ markedly from this ratio. Hamlin and Valencia oranges show a relatively high linolenic acid percentage of 16.4 and 16.2, respectively, and concomitantly lower oleic acid. The Temple tangor shows the greatest departure from this ratio, i.e., it consists of 34.2% linolenic acid and concomitantly lower percentages of oleic and linoleic acids. The high percentage of 18:3 for Hamlin and Temple triglycerides is consistent with the high 18:3 found for total fatty acids in these two varieties (5); however, the 18.3 percentage in Valencia triglyceride is not consistent with its total fatty acid profile (4,5). Fatty acids longer than 20:1 were detected in these triglycerides but were never detected at percentages greater than 0.1% and therefore were excluded from this Table.

The fatty acid composition of MGDG is shown in Table II. The five major acids are found in an approximate ratio of 0.5:1:3:1.5:4 and comprise between 96.5% and 98% of all acids. MGDG shows a markedly higher percentage of 18:3 than found in triglycerides, steryl esters and esterified steryl glucosides. Parson Brown and Valencia possess the lowest percentage of 18:3 while the two tangors, Temple and Temple x Kinnow, show very high percentages of 44.6 and 47.1, respectively. The average percentage of 18:3 found in the eight varieties is similar to potato MGDG (12) but far less than apple MGDG (16). In citrus MGDG, the ratio of 16:0 to 16:1 is ca. 1:2 for Walker Early, Parson Brown, Washington Navel and Pineapple; and is ca. 1:1 for Hamlin, Valencia, Temple and Temple x Kinnow. The relative concentration of 16:1 is in the range of 6-11%. This is in contrast to MGDG in potato (12), corn (14) and apple (16) where this acid is not even a constituent. The average percentage of 18:2 in citrus MGDG is 16.3. This percentage is quite close to the 12.7% reported for apple (16)

TABLE III

Fatty Acids of Steryl Esters From Orange and Tangor Juice Sacs, %

Carbon No.	Walker Early[a]	Parson Brown	Hamlin	Washington Navel	Pineapple	Valencia	Temple	Temple x Kinnow
12	1.17 ± .15	0.72 ± .06	0.83 ± .07	0.48 ± .08	0.90 ± .15	0.77 ± .08	1.19 ± .11	1.31 ± .10
14	3.80 ± .43	2.14 ± .11	2.78 ± .19	1.69 ± .25	2.27 ± .19	1.71 ± .14	2.26 ± .18	3.21 ± .13
16	7.11 ± .33	4.82 ± .24	7.02 ± .17	4.97 ± .33	6.01 ± .18	5.81 ± .32	4.31 ± .31	8.18 ± .21
16:1[b]	5.26 ± .31	5.27 ± .43	6.29 ± .45	5.96 ± .30	5.07 ± .35	3.69 ± .22	4.44 ± .12	5.31 ± .20
16:2 + I 18[c]	0.38 ± .05	0.46 ± .07	0.29 ± .05	0.46 ± .06	0.43 ± .14	0.43 ± .05	0.26 ± .04	0.30 ± .12
18	1.50 ± .05	0.90 ± .07	1.17 ± .09	1.05 ± .08	1.17 ± .14	1.10 ± .11	0.88 ± .04	1.39 ± .10
18:1	16.57 ± .30	16.14 ± .41	14.82 ± .35	17.98 ± .54	16.59 ± .88	15.46 ± .40	16.97 ± .13	19.25 ± .34
18:2	30.17 ± .58	42.33 ± .44	40.48 ± .90	39.50 ± .25	43.71 ± .45	35.57 ± .63	19.75 ± .43	40.05 ± .74
18:3	7.76 ± .33	10.81 ± .28	14.54 ± .55	12.94 ± .41	10.90 ± .35	9.61 ± .24	31.31 ± .48	7.29 ± .21
20:1	0.61 ± .14	0.56 ± .16	0.29 ± .05	0.58 ± .14	0.60 ± .14	0.67 ± .09	0.89 ± .09	0.56 ± .08
22	2.02 ± .20	1.45 ± .19	1.16 ± .11	1.17 ± .18	1.22 ± .25	1.86 ± .11	1.49 ± .27	1.05 ± .11
23	1.47 ± .27	0.96 ± .11	0.66 ± .19	0.89 ± .19	0.65 ± .14	1.30 ± .11	1.07 ± .23	0.62 ± .11
I24	1.79 ± .24	1.26 ± .20	0.58 ± .13	0.92 ± .13	0.57 ± .08	0.84 ± .04	0.78 ± .09	0.60 ± .08
24	10.34 ± .54	5.96 ± .23	5.27 ± .42	6.05 ± .13	5.39 ± .34	10.31 ± .29	8.10 ± .28	5.11 ± .23
25	2.83 ± .19	1.76 ± .17	1.25 ± .14	1.63 ± .16	1.38 ± .17	3.18 ± .18	1.69 ± .18	1.67 ± .11
I26	1.72 ± .34	1.21 ± .20	0.39 ± .08	0.98 ± .12	0.61 ± .08	1.16 ± .15	1.06 ± .08	1.27 ± .11
26	5.49 ± .18	3.25 ± .27	2.18 ± .34	2.75 ± .19	2.53 ± .12	6.53 ± .14	3.55 ± .32	2.83 ± .23

[a]Mean ± SD of nine determinations.

[b]Number of double bonds.

[c]Unresolved peak of 16:2 and iso 18.

but less than the 58% reported for potato (12) and 36.4% for corn (14). As was the case for triglyceride, no fatty acid longer than 20 carbons could be detected at percentages greater than 0.1% in any variety.

The fatty acid profile of steryl esters varies considerably from variety to variety as observed in Table III. Generally the approximate ratio for the five major acids is 1:1:3:7:2. The Walker Early orange and Temple x Kinnow tangor show the lowest percentage of 18:3 while the Temple (31.3%) shows a percentage approximately three times greater than that of all other varieties. This large percentage of 18:3 for Temple is accompanied by a relatively lower 18:2. The five major acids, 16:0, 16:1, 18:1, 18:2 and 18:3, constitute greater than 96% of the acids in triglycerides and MGDG, however, these acids in steryl esters constitute between 66.9% and 83.2% of the total acids. Accounting for the lower concentrations of these five major acids is the occurrence of long chain acids in the range 22:0 to 26:0 at relative concentrations up to 10.3% (24:0 in Valencia). These long chain acids are found at relative concentrations of 24:0>26:0>25:0>22:0>23:0. Two iso-branched acids are also found in this region, iso 24:0 and iso 26:0, at a concentration range of .39% to 1.79%. The ratio of 22:0, 24:0 and 26:0 fatty acids found for total citrus lipids (5) is 1:2:1, respectively, however in these steryl ester profiles this ratio is 1:5:2. One striking exception to this ratio is found in Valencia where the ratio is 2:6:3 in total Valencia lipid (5) and 1:5:3 for its steryl ester. Of the four different lipids analyzed, the steryl ester group is the only one to show the 15:0 acid in concentrations less than 0.1% and, therefore, this acid is not reported in this Table. Another characteristic feature of steryl esters is the presence of noticeable amounts of two iso-branched acids, iso 24:0 and iso 26:0.

Table IV shows the fatty acid composition of ESG. The five major acids constitute between 91.5% and 95.3% of the total acids and their approximate ratio is 3.5:1.5:5:2:1. Palmitic and oleic acids are found at the highest concentration in this lipid. This large percentage of 16:0 is characteristic of ESG (12,15). ESG also shows the highest ratio of 16:0 to 16:1. The concentrations of palmitic and oleic acid show noticeable variations between varieties while the percentage of palmitoleic and linoleic acid are relatively constant. Only one variety, Temple, has an 18:2 content less than half that found for the other varieties. Temple and Valencia manifest quantities of 18:3 greater than twice the average found for the other six varieties. In contradistinction to steryl esters, ESG shows the presence of 15:0 above the trace level; however, the two iso acids, iso 24:0 and iso 26:0, are not found in any variety above the trace level and therefore, are exluded from this Table.

A study of the fatty acid profiles of specific lipid constituents of citrus has shown that fatty acid patterns can be used in chemotaxonomic differentiation. There is no doubt that this study could be extended to other citrus varieties at similar or different times of maturity. Valencia was the only late season orange variety examined in this study. The fatty acid profiles of the four Valencia lipid constituents manifests differences which may be employed to differentiate this variety from the other. Pineapple, the only midseason orange variety examined, can be shown to differ from other citrus varieties as follows: from Hamlin and Valencia in its 18:3 triglyceride content; from Walker Early and Temple in its 18:2 steryl ester content from Parson Brown, Temple x Kinnow, in its 18:3 MGDG content; and from Washington Navel in its 18:2 MGDG content. Four early season orange varieties were studied. A most outstanding characteristic which distinguishes Walker Early from the other three early orange varieties is its high 18:3 content in ESG. Parson Brown has a lower 18:3 in MGDG, Hamlin, a higher 18:3 in triglyceride and Washington Navel a lower 18:2 in triglyceride than that manifest by the other three early season varieties. The Temple tangor has fatty acid profiles different from the other seven citrus varieties in all four lipid constituents. In all four of these lipids Temple can be characterized with one exception as having a much higher 18:3 content. This one exception is the MGDG of the Temple x Kinnow tangor which has a higher 18:3 content, i.e., 47.1% vs. 44.6% for Temple. Whether this higher 18:3 percentage is due to the *C. reticulata* parentage is still to be investigated.

The observation that longer chain fatty acids in the 22:0 to 26:0 range are associated mainly with steryl ester lipid of juice sacs is very interesting. The biological function of these lipids is not understood since it has not been proven that steryl esters are transported as such from one tissue to another in plants (17). Like other plants (12,16), citrus has a high percentage of linolenic acid in its MGDG lipids. Unlike other plants, however, it has the unique property of containing appreciable amounts of palmitoleic acid. Citrus esterified steryl glucosides like other plant ESGs have a high palmitic acid content. Unlike other plant lipids, the stearic acid content is relatively low (1% to 1.5%). This low stearic acid content has been previously

TABLE IV

Fatty Acids of Esterified Steryl Glucosides from Orange and Tangor Juice Sacs, %

Carbon No.	Walker Early[a]	Parson Brown	Hamlin	Washington Navel	Pineapple	Valencia	Temple	Temple x Kinnow
12	0.23 ± .03	Trace[b]	0.24 ± .05	0.28 ± .07	0.21 ± .06	0.22 ± .01	0.48 ± .09	0.38 ± .02
14	1.24 ± .06	0.50 ± .05	0.64 ± .08	0.47 ± .05	0.42 ± .06	0.83 ± .07	1.38 ± .08	0.95 ± .27
15	0.47 ± .04	0.29 ± .03	0.47 ± .07	0.36 ± .05	0.30 ± .08	0.64 ± .05	0.78 ± .06	0.42 ± .06
16	21.97 ± .52	26.24 ± .60	29.88 ± .44	23.25 ± .74	25.85 ± .32	30.35 ± .39	26.21 ± .50	22.37 ± .60
16:1[c]	8.05 ± .49	7.59 ± .26	4.67 ± .26	8.57 ± .27	6.42 ± .41	5.10 ± .32	7.17 ± .06	6.41 ± .26
16:2 + I 18[d]	0.53 ± .07	0.55 ± .04	0.50 ± .06	0.49 ± .08	0.60 ± .09	0.52 ± .02	0.50 ± .09	0.49 ± .02
18	1.23 ± .03	0.91 ± .14	1.33 ± .13	0.79 ± .07	0.86 ± .04	1.01 ± .11	1.38 ± .14	1.19 ± .09
18:1	37.65 ± .52	40.78 ± .43	36.05 ± .96	42.46 ± .68	41.29 ± 1.00	33.74 ± .89	37.17 ± .64	41.77 ± .65
18:2	15.38 ± .21	16.30 ± .85	18.43 ± .24	15.81 ± .50	16.66 ± .36	19.29 ± .50	6.72 ± .23	18.64 ± .27
18:3	11.01 ± .87	4.28 ± .21	6.26 ± .29	5.07 ± .04	4.82 ± .13	5.82 ± .20	14.18 ± .69	4.99 ± .29
20:1	0.73 ± .08	0.48 ± .11	0.27 ± .07	0.42 ± .10	0.39 ± .03	0.26 ± .08	0.45 ± .05	0.46 ± .09
22	0.44 ± .05	0.50 ± .07	0.56 ± .13	0.56 ± .18	0.50 ± .03	0.45 ± .08	0.66 ± .08	0.66 ± .06
23	0.14 ± .04	0.25 ± .07	0.12 ± .03	0.19 ± .04	0.15 ± .03	0.15 ± .03	0.44 ± .02	0.19 ± .04
24	0.71 ± .05	0.90 ± .14	0.58 ± .07	1.01 ± .13	1.09 ± .10	0.96 ± .14	1.65 ± .15	0.83 ± .05
25	0.10 ± .03	0.13 ± .01	Trace	0.20 ± .09	0.16 ± .02	0.26 ± .05	0.50 ± .07	0.14 ± .04
26	0.12 ± .06	0.30 ± .03	Trace	0.27 ± .09	0.28 ± .04	0.40 ± .08	0.33 ± .10	0.11 ± .04

[a]Mean ± SD of nine determinations.

[b]Trace, less than 0.1%.

[c]Number of double bonds.

[d]Unresolved peak of 16:2 and iso 18.

reported (4) to be characteristic of citrus species.

All eight varieties of fruit were from the 1969-1970 season. A question may arise whether fruit from previous seasons would have different fatty acid profiles. An examination of the profiles of Valencia steryl ester and esterified steryl glucoside from the 1968-1969 season (H.E. Nordby and S. Nagy, unpublished data) and those from the 1969-1970 season showed very close agreement.

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Wax Esters in Fish: Turnover of Oleic Acid in Wax Esters and Triglycerides of Gouramis

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ABSTRACT

Methyl U-^{14}C oleate was fed to mature male and female gouramis (*Trichogaster cosby*) and the radioactivity in lipids measured over a period of four months. Initial incorporations were 70-80% and more than half of that was still in the lipids at the end of the experiment. Very little conversion of the 18:1 chain had occurred. Main storage of the labeled 18:1 chain was in the wax esters of the roe and in the triglycerides of the body. In the wax esters, 18:1 occurred in both the alcohol and acid moieties. Initially the females had less radioactivity in the triglycerides than in the wax esters but at the end of the experiment this was reversed. An appreciable amount of 18:1 had been transferred from roe to body lipids. The biological half life of 18:1 in gouramis is estimated to be about four months. This time is equal for males and females although translocation from roe to body and transformation of wax ester to triglyceride take place in the female, whereas wax esters do not play any role in the lipid metabolism of the male.

INTRODUCTION

Fish generally store energy in the form of triglycerides, but some species use wax esters for this purpose (1-3). Gouramis and several other species exhibit both types of storage in the course of their life cycle (4,5). Wax esters are prominent in the roe and the very young fry, but they are replaced by triglycerides as the newly hatched fish uses up the supplies of the egg sac and takes external food. Wax esters are a very minor lipid component in the mature males. Only the females accumulate wax esters again in the roe.

Opaline gouramis (*Trichogaster cosby*) were well adaptable to laboratory experimentation and it was shown that incorporations of dietary 16:0 and 18:1 acids and alcohols were between 30% and 80% (6, D.M. Sand, J.L. Hehl and H. Schlenk, manuscript in preparation). Corresponding acids and alcohols were readily interconverted by the fish, but very little conversion of the chains took place. Likewise, resynthesis from catabolized chains was only minor.

These observations were from experiments over a period of only 24 hr. In the work reported here the metabolism of the 18:1 compounds and the interrelations of body and roe lipids were studied for longer periods. The level of ^{14}C, given as methyl [U-^{14}C] 18:1 ester to female gouramis was checked in an exploratory experiment for a period of two months, but no significant decrease of radioactivity in the fish was detected by the end of that time. Therefore, subsequent experiments, which are described here, were carried on for four months. After this period, more than half of the ^{14}C that originally had been incorporated by the fish was retained in the lipids. About 90% of the radioactivity was still in 18:1 chains and 10% was found in other chains. In females, an appreciable amount of [U-^{14}C] 18:1 that initially had been deposited in roe wax esters had been transported to body triglycerides.

TABLE I

Composition of Fish Food[a]

Acid[b]	Per cent	Acid[b]	Per cent
14:0	3.8	18:2ω6	15.6
16:0	16.9	18:3ω3	5.8
16:1	4.6	20:4ω6	4.9
18:0	7.1	20:5ω3	6.4
18:1	20.0	22:6ω3	8.7

[a]Shrimp-el-ettes, labeled "Crude protein not less than 37%" and "Crude lipid not less than 5%." Extraction with $CHCl_3/CH_3OH$ yielded 10.3% lipids from which 7.5% fatty acids was obtained.
[b]Minor components are omitted.

EXPERIMENTS AND RESULTS

Sets of 24 male and 18 female gouramis, 9 and 10 months old, respectively, were taken from our stock colony and were kept in groups of three in five-gallon tanks (6). Water was changed at least every two weeks. The fish were trained for feeding and, together with their normal diet, were offered 10.0 mg methyl [U-^{14}C] oleate (8.6×10^6 or 5.3×10^6 dpm) (8) per tank of three specimens at the beginning of the experiments. The diet consisted of 900 mg dry food (Shrimp-el-ettes, Longlife Fish Food Products, Harrison, N.J.) daily per tank before

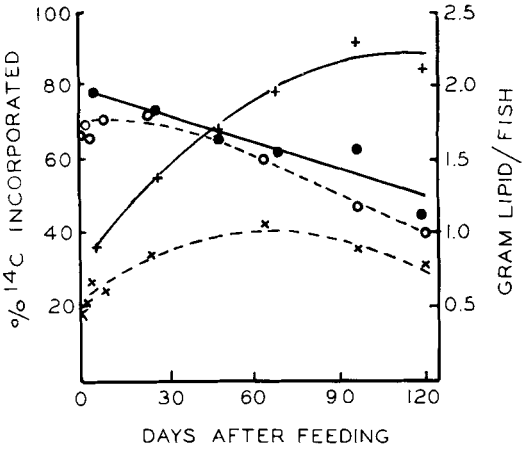

FIG. 1. Lipid amount and incorporation of ^{14}C-labeled oleic acid in gouramis. Age of females at the beginning of the experiment, 10 months, of males, 9 months. Symbols: ————, female; +, g lipids per fish; •, per cent of ^{14}C offered in these lipids; - - - -, male; x, g lipids per fish; ○, per cent of ^{14}C offered in these lipids.

and during the experiment. The food is described in Table I. Most of it was eaten within 20 min. At time intervals the three fish from one tank were sacrificed. Lipids of body, roe, liver and intestines with stomach and adhering fat were extracted and analyzed separately according to procedures already described (6,7).

Figure 1 shows the weight of lipids per fish for males and females and their content of ^{14}C in percentages of the amount offered. Weight proportions of lipids from different parts of the fish did not indicate any significant trend over the four-month period of the experiment. In males, the liver lipids were ~2%, intestinal lipids ~15% and body lipids ~80% of the total lipids; in females, the liver lipids were 1-2%, intestinal lipids ~10%, body lipids 30-40% and roe lipids 40-50% of the total lipids. The distribution of radioactivity among these lipids of the females is given in Table II.

TABLE II

Distribution of ^{14}C Among Lipids of Female Gouramis[a]

^{14}C in Lipids of	Days after feeding					
	5	26	48	68	96	118
Body	16.6	19.3	22.1	23.7	29.7	26.0
Roe	55.4	45.3	36.6	32.8	25.4	11.8
Intestines	5.1	7.6	5.5	4.2	7.0	5.5
Liver	0.4	0.3	0.6	0.3	0.3	0.3
	77.5	72.5	64.8	61.0	62.4	43.6

[a]Per cent of radioactivity offered.

The classes of lipids from body and from roe of females were separated by column chromatography on silicic acid (6), weighed and counted. Triglycerides and wax esters represented 80-90% of the body and roe lipids, respectively. The radioactivities of these fractions are given in Table III. Methanolysis of the triglycerides with HCl in methanol yielded fatty acid methyl esters and methanolysis of the wax esters with BF_3 in methanol gave methyl esters and fatty alcohols (6). The latter products were separated by thin layer chromatography (TLC) and the alcohols were acetylated. The three type esters were fractionated according to chain length by preparative gas liquid chromatography (GLC) and the radioactivity of each fraction was determined (Table IV).

As one might expect, conversions were relatively highest after the longest time period. Therefore, only the four-month samples were subjected to more detailed analysis to determine the distribution of radioactivity among individual acids and alcohols (Table V). The

TABLE III

^{14}C in Triglycerides and Wax Esters of Female Gouramis

Days after feeding	Per cent ^{14}C of body lipids in triglycerides[a]	Per cent ^{14}C of roe lipids in wax esters	wax alcohols / wax acids
5	86.8[b]	95.6	30.3 / 65.4
48	92.9	96.1	32.9 / 63.2
96	97.4	96.5	29.0 / 67.5
118	97.7	95.5	35.3 / 60.2

[a]Virtually all ^{14}C is in the fatty acids.
[b]Lecithins contained 6.3% of the ^{14}C in body lipids after five days, but less ^{14}C after longer periods.

TABLE IV

Distribution of ^{14}C Among Chains

Sample	Days after feeding	Per cent ^{14}C in[a] C_{16}	C_{18}	C_{20}
Female	5	2.3	94.5	2.8
Alcohols from	48	3.6	90.9	4.6
wax esters	96	5.1	89.5	4.1
	118	7.3	86.1	4.6
Acids from	5	1.4	97.6	0.7
wax esters	48	1.9	96.7	0.9
	96	2.5	95.6	1.2
	118	3.6	93.3	1.8
Acids from	5	0.9	97.9	0.9
body triglycerides	48	1.4	97.0	1.2
	96	1.8	96.2	1.5
	118	2.5	95.1	1.8
Male	120	1.7	96.1	1.4
Acids from body triglycerides				

[a]^{14}C contained 0.1-0.5% and C_{22} 0.2-1.2% of the radioactivity.

fractions of equal chain lengths from preparative GLC were separated according to unsaturation by chromatography on glass fiber sheets impregnated with SiO_2 (Type SA, Gelman Instrument Co., Ann Arbor, Michigan) and $AgNO_3$ (9) using the solvent system, hexane + diethyl ether + acetic acid, 95:5:0.5. The spots were located by the dichlorofluorescein-UV method; the chromatograms were cut accordingly and the increments counted (7) with recoveries of counts being >90%. The values for spots of individual esters were compared by reference to the specific activity of respective GLC fractions to calculate the distribution of ^{14}C among all components of the mixture (Table V). The same procedures were applied to the body triglycerides of the males. The pertinent results from these triglycerides are given at the bottom of Tables IV and V.

DISCUSSION

Opaline gouramis are mature for reproduction at the age of about 10 months, but neither growth nor accumulation of fat have reached their limit by that time. According to Figure 1, males reached their maximum amount of fat at the age of about 11 months (9 months + 60 days) and females at 14 months (10 months + 120 days). However, these observations cannot be generalized for the opaline gourami. Individuals from the same hatch represent a great variety of sizes. The fish used in our experiments had been selected for equal size, but they certainly were genetically heterogeneous. Moreover, growth of gouramis, as of other fish, depends greatly on temperature, size and population of the tank and doubtlessly on the amount and quality of food. Such factors were kept as equal as possible.

The radioactivities decreased steadily but so slowly that the fish had retained, after four months, still more than 50% of the ^{14}C initially incorporated (Figure 1). This applies to both males and females although the former had lost some of their lipids, whereas, in the latter, the amount of lipids increased over the whole period.

As in previous experiments, the female

TABLE V

Distribution of ^{14}C Among Components After 118 Days[a]

Sample	Per cent ^{14}C in structure 16:0	16:1[b]	18:0	18:1[b]	20:0	20:1[b]
Female						
Alcohols from wax esters	4.8	2.2	0.95	84.6	—	3.2
Acids from wax esters	0.2	3.1	0.1	92.4	0.1	1.3
Acids from body triglycerides	1.0	1.4	0.1	94.8	0.1	1.3
Acids from total intestinal fat[c]	0.8	2.2	0.7	92.6	0.2	2.2
Male[d]						
Acids from triglycerides	0.7	0.9	0.1	95.4	<0.1	0.9

[a]Chemical analyses of acids and alcohols from lipid classes of gourami are given in References 4, 6 and 7.

[b]^{14}C 0.1-1.0% was found in the area of polyunsaturated C_{16}, C_{18} and C_{20} acids or alcohols.

[c]Mainly triglycerides.

[d]Males were sacrificed 120 days after feeding.

gourami initially incorporated more ^{14}C in roe than in body lipids (Table II) but by the end of the experiment the situation is reversed. The balance of ^{14}C after four months shows loss from roe (45%), increase in body (10%), and overall loss by catabolism (35%), all in reference to the amount offered. The transfer of 10% ^{14}C from roe to body is a minimum figure. The amount actually translocated must be larger since it is very unlikely that body depot lipids are excepted from catabolism.

The absolute increase of ^{14}C in body lipids must be explained by reabsorption of lipids from overmature eggs which have not been extruded. When eggs are not discharged, their turnover is fast enough to supply considerably more lipids than required for energy. Some of these lipids are deposited in the body and possibly also in new developing eggs diluted with dietary and endogenous fat.

The same shift of radioactivity from roe to body had been encountered in the first exploratory experiment, although there the loss of ^{14}C by catabolism had not become as obvious after two months.

Extrusion and eating of eggs has never been observed with female gouramis in absence of males. Moreover, such external recycling of radioactive wax esters would rather maintain than change the ratio of ^{14}C in lipids of roe and body. When radioactive wax was fed (unpublished experiments) the ^{14}C balance was established in favor of the roe as it had been with dietary ^{14}C labeled alcohols and acids (6).

The wax esters and triglycerides contain ~95% of the ^{14}C (Table III) and are composed largely of saturated and monounsaturated structures (7). The distribution of radioactivity among the chains (Tables IV and V) shows that ^{14}C is slowly dissipated to other structures, but more than 90% of 18:1 remains unchanged. De novo synthesis by recycling of ^{14}C through the acetate pool is most obvious with the alcohols in which 16:0 represents 50% of all components. Nevertheless, 16:0 has only 5% of the total radioactivity in alcohols. Synthesis from radioactive acetate pertains also to 18:1 chains, which then cannot be distinguished from those originally labeled with ^{14}C. However, it seems unlikely that this would take place to an appreciable extent without considerable increase of radioactivity in 16:0. The data of Table V also indicate some elongation of 18:1 to 20:1 and partial degradation to 16:1. Wax esters and triglycerides contain much more of 16:0 than 16:1 components and the relatively high radioactivity in the latter likely is due to partial degradation of 18:1.

It appears that 18:1 alcohol and acid of wax esters are metabolized at a very similar rate. With corrections applied for radioactivity in other chains (Table IV or V) the ratio of ^{14}C in 18:1 alcohol-acid (Table III) changes from 28.6:63.8 at the beginning to 29.9:55.7 after 118 days. The slight increase may indicate a slightly lower metabolic rate for the alcohol.

More than half of the radioactivity has been retained by the gouramis over the whole period of 118 days (Fig. 1 or Table II). Most of this radioactivity is still in C_{18} chains (Table IV), i.e., in 18:1 since radioactivity of 18:0 at the end of the experiment is negligible (Table V). When calculated from data of Tables II-IV, the sums of radioactivity in C_{18} chains of wax esters and body triglycerides of females, are 63.1%, 51.9%, 41.1% and 31.3% of the amount offered, at 5, 48, 96 and 118 days. This represents a regression by about 50%. Omission of other lipid classes can involve only small error since their radioactivity is very minor. Therefore, the half life of the 18:1 structure in gouramis is approximately four months. In the female the time is somewhat shorter in the roe, but this is due to translocation rather than catabolism. With the continuous supply from the roe, the half life time appears longer in the body lipids. In total, the female metabolizes 18:1 at least as economically as the male although reduction, oxidation and transport from roe to body are involved when eggs are not deposited.

It is generally accepted that the poikilothermic fish have slower rates of metabolism than homeothermic vertebrates. For comparison, it may be quoted that mice and rats burn dietary oleic acid to 50% within 16 to 20 hr (10,11), which is about 150 times faster than the catabolism of this structure in gouramis.

ACKNOWLEDGMENTS

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The Desaturation Step in the Animal Biosynthesis of Polyunsaturated Fatty Acids

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ABSTRACT

The aerobic desaturation of unsaturated fatty acids in the microsomes has been systematically explored and some of the relevant experimental works have been assembled. The contribution of the microsomal electron transport chain and lipoproteic structure of the microsomes is analyzed. Evidences of linoleyl-CoA and α-linolenyl-CoA being desaturated by the same enzyme are presented. The linoleic acid desaturation is shown to be different in different tissues and to decrease with aging. The effect of competitive reactions with acids of the same or different series, the competition of desaturation and transesterification, and dietary and hormonal contributions to unsaturated fatty acids desaturation are summarized. All *trans* linoleic acid and elaidic acid were not desaturated in our experimental conditions by rat liver microsomes. From the bulk of data collected, a hypothetical model of 6-olefinase is drawn. The main features of the model are: The existence of both binding and desaturating sites; the binding of acyl-CoA and enzyme through hydrophobic forces of the Van der Waals type and weak polar attractions due to double bonds; the orienting binding characteristics of double bond proximate to the place where olefination will take place; and the importance of enzyme conformation that requires that the orienting double bond must have a *cis* structure.

INTRODUCTION

The biosynthesis of polyunsaturated fatty acids in the animals is produced by aerobic desaturations and elongations of fatty acids previously formed or provided in the food. These reactions take place in the microsomes. The scheme of the different and possible sequences of reactions that may take place in the biosynthesis of the three main fatty acid series is shown in Figure 1. However, in spite of the different possible pathways that have been shown, the preferred routes appear to be alternative desaturations and elongations. Marcel et al. (1) have shown in this respect that the dominant pathway for arachidonic acid

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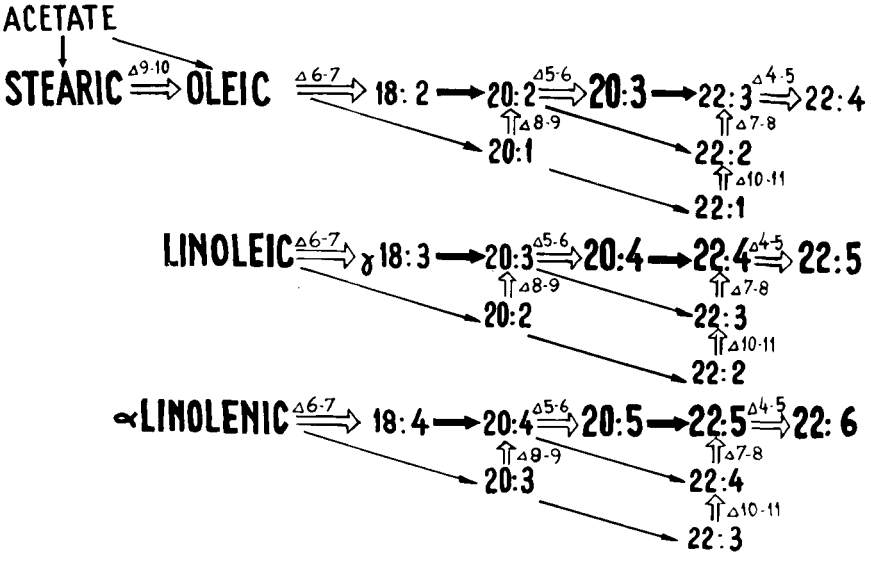

FIG. 1. Different pathways in the animal biosynthesis of oleic, linoleic and α-linolenic acid series.

TABLE I

Effect of Different Nucleotides Upon the Conversion of Free Linoleic Acid to γ-Linolenic Acid

Nucleotide	Conversion, %
ATP	11.0
GTP	12.9
CTP	10.9
AMP	3.3
-------	0.0

[a]Five milligrams of microsomal protein incubated 20 min at 35 C in air with 5 nmoles -1-^{14}C-linoleic acid; 2.5 μmoles nucleotide and other cofactors in 3 ml at pH 7.0.

biosynthesis follows this type of sequences.

It is interesting to remark that in spite of the different positions of the double bonds in the three fatty acid families, there are remarkable similarities, and for a given chain length the new double bonds are opened at the same distance of the -COOH in all the series (Fig. 1). However, the yield of the reactions is quite different for each acid when measured in vitro as it has been shown repeatedly (2).

In this paper, a review of the different results we have obtained in the desaturation of fatty acids will be compared with new findings and a model of the 6-desaturase will be proposed to explain them.

MATERIALS AND METHODS

Cis unsaturated fatty acids 1-^{14}C were purchased from the Radiochemical Centre, Amersham, England. They were 98% radiochemically pure. All *trans* linoleic acid 1-^{14}C (52.5 mC/mmole, 99% radiochemical purity) was purchased from Applied Science Lab., Inc, State College, Pa. 1-^{14}C Elaidic acid (58 mC/mmole) was provided by the Radiochemical Centre, Amersham, England. It was purified by thin layer chromatography in silica gel impregnated with silver nitrate. Unlabeled acids were provided by the Hormel Institute, Austin, Minn. All of them were 99% pure. Cofactors were purchased from Fluka A.G., Buchs, Switzerland.

Separation of Microsomes

Male albino rats from the Institute strain were used. After killing the animals the livers were immediately homogenized in the cold with 0.15 KC gluthathione, 62 mM phosphate buffer (pH 7) and 0.25 M sucrose. The microsomes were separated by differential centrifugation in a Spinco L2 ultracentrifuge and 140,000 x *g* in the usual way (2).

Measurement of Fatty Acid Desaturation

Three procedures were used. The per cent conversion was measured incubating 5 mg microsomal protein with at least 5 nmoles labeled acid for 20 min at 35 C in air. Three millilitres of the incubation medium contained, in micromoles: 2.5 ATP, 0.2 CoA, 2.6 NADH, 15 $MgCl_2$, 4.5 glutathione, 1 nicotinamide 125 NaF and 125 phosphate buffer (pH 7.0) in 0.15 M KCl and 0.25 M sucrose. The incubation was stopped by adding 10% methanolic KOH. The fatty acids were converted to the methyl esters. The distribution of radioactivity between substrate and product was measured by gas liquid radiochromatography in a Pye apparatus with a proportional counter under the conditions described in a previous work (2).

The approximate velocity of the desaturation of the free acid was measured by a similar procedure, but incubation was stopped after 4 min.

In another group of experiments, instead of incubating the free acids, the CoA derivatives were used. In these conditions, the acylation of the CoA was avoided. The incubation medium contained increasing amounts of acyl CoA, 0.5 to 5 mg microsomal protein, 1 μmole NADH

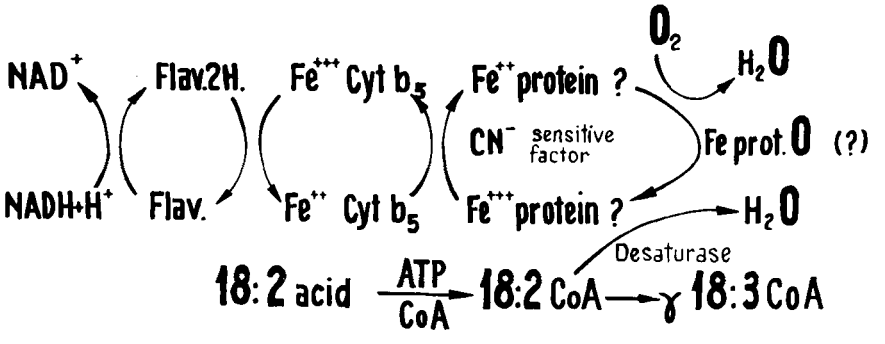

FIG. 2. Hypothetical scheme of microsomal electron transport chain coupled to linoleic acid oxidative desaturation.

and 30 μmoles phosphate buffer (pH 7.2) in a total volume of 0.5 ml. The reaction was stopped after 4 min of incubation at 35 C. The amount of the product labeled with ^{14}C was measured by gas liquid radiochromatography in the usual way (2).

RESULTS AND DISCUSSION

Acylation of CoA

The desaturation of fatty acids requires a previous activation to an acyl-CoA. In this activation the energy is generally supplied by ATP conversion to AMP and pyrophosphate (3). However, we have found that liver microsomes may convert free linoleic acid to γ-linolenic acid in the presence of NADH, CoA and Mg^{++} by either ATP, GTP or CTP (Table I). This result at first instance would suggest that, directly or indirectly, CTP and GTP would be able to provide the necessary energy to convert linoleic acid to linoleyl-CoA. (R.R. Brenner and A. Catala, submitted for publication).

Microsomal Electron Transport Chain

The desaturating enzymes of unsaturated fatty acids are tightly bound to the encoplasmic reticulum and require 0_2 and NADH or NADPH, NADH being a little more effective than NADPH.

The microsomal electron transport system is involved in the reaction. For some time was called the fatty acid desaturating enzyme "oxygenase" (2) and afterwards, more vaguely, "desaturase." Today we prefer and would suggest to call it "olefinase," considering that it produces an olefination of the hydrocarbon tail of the fatty acid. First we called it "oxygenase," following Hayaishi's nomenclature (4) (it would correspond to a "mixed function oxidase" of Mason), considering that it uses NADH and 0_2. However, in the classical microsomal monoxygenases of Hayaishi, also called hydroxilases, the mechanism of reaction involves the use of cytochome P 450 and the synthesis of hydroxilated compounds (4). But in the fatty acid olefination, as far as it is known, no intermediate oxygenated products have been definitively isolated and besides Imai and Sato (4) showed that cytochrome P450 is not used in fatty acid olefination. For this reason, this name is not used widely.

The microsomal nonphosphorilating electron transport chain involved in the fatty acid desaturation includes a flavoprotein, a cyanide sensitive factor (5,6) attributable to an hemoprotein (7) and a cytochrome b5 (8). A hypothetical scheme of the microsomal electron transport system coupled to the linoleyl-CoA desaturation is shown in Figure 2.

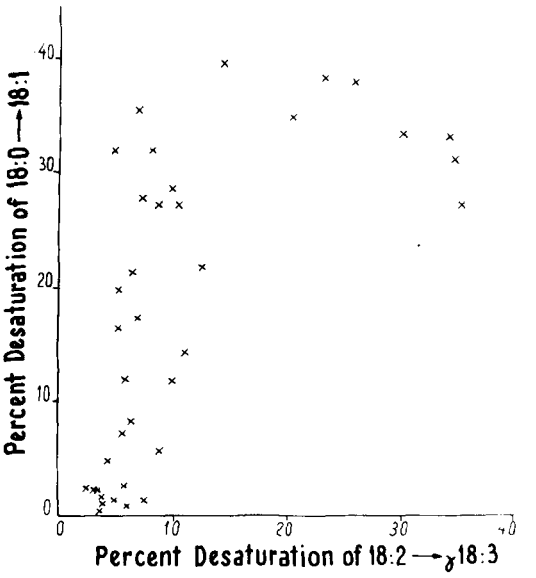

FIG. 3. Relationship between linoleic acid and stearic acid desaturation in rat liver microsomes.

Necessity of Lipids

The activity of the electron transport chain of the microsomes depends on a lipoproteic structure (9). At least a similar situation is postulated by Green and Tzagoloff (10) for the mitochondrial electron transport chain located in the inner membrane. Amphipatic lipids, like phospholipids, may associate to cytochrome and at the same time to the membrane proteins. Besides, the microsomal vesicules formed principally from the endoplasmic reticulum by disruption and differential centrifugation at 100,000 x *g* are rich in polar and nonpolar lipids (11,12). Jones et al. (9), have shown that acetone extracted microsomes lose their olefinating properties. These properties are recovered by addition of a proper mixture of phospholipids, triglycerides and fatty acids.

Therefore, these results suggest that the fatty acid desaturating activity of the microsomes is highly dependent on the integrity of a lipoproteic structure. For the moment, the innumerable intents to prepare a soluble unsaturated fatty acid olefinase from animal sources have been unsuccessful. Only Gurr et al. (13, 14) have been able to prepare a "soluble" stearoyl-CoA olefinase from liver microsomes by gentle homogenization in 1 M phosphate buffer pH 7.4 at 0 C. However, it is necessary to emphasize that this so called "soluble" enzyme is still bound to fairly large aggregates,

TABLE II

Differentiation of Stearic From Linoleic and α-Linolenic Acid Desaturation by Liver Microsomes in Vitro

	Stearic 18:0 → 18:1	Linoleic 18:2 → γ-18:3	α-Linolenic α-18:3 → 18:4
Solubilization of the desaturase by Gurr et al. method (14)[a]	+		Not tested
Correlation of activities	With 18:2- none With α 18:3- none	With 18:0- none With α 18:3-+	With 18:0- none With 18:2- +
Dietary effects Fasting (17)	Decrease	Decrease[b]	Decrease[b]
Glucose after fasting (17)	Increase	Increase[b]	Increase[b]
Protein (15, 16)	No effect	Increase	Increase
Alloxanic Diabetes (18)	Decrease	Decrease[b]	Decrease[b]
Insulin (18)	Increase	Increase[b]	Increase[b]
Preincubation of microsomes with ATP[c]	Insensitive	Increase	Increase

[a]O. Mercuri, personal communication.

[b]Less sensitive.

[c]R.R. Brenner and A. Catala, submitted for publication.

containing at least those enzymes participating in the electron transport system associated with fatty acid desaturation and with steroid hydroxylation (13,14).

Differentiation of Stearoyl-CoA Olefinase From Linoleyl-CoA Olefinase

Enough information has been collected today to consider that liver stearoyl-CoA olefinase is different from linoleyl-CoA olefinase. From kinetic data, Brenner and Peluffo (2) have proposed in 1966 that very probably stearic acid 9-olefinase was different from linoleic acid 6-olefinase and that very probably 6-desaturation of oleic, linoleic and α-linolenic acid was produced by the same enzyme. From dietary studies, Inkpen et al. (15) also proposed that α-linolenic acid 6-olefinase was different from stearic acid 9-olefinase. Besides, results obtained by Inkpen et al.(15) show a remarkable similarity with linoleic acid 6-desaturation response to similar dietary variation, found by Gomez Dumm et al (16). A lack of correlation between linoleic and stearic acid desaturation was also found when the conversions of both acids were measured simultaneously in the same microsomes of rats with different hormonal or dietary treatments (Fig. 3). A summary of the different responses of stearic, linoleic and α-linolenic acid desaturation under different stimuli and conditions is shown in Table II. These results would indicate that 6-olefinase and 9-olefinase are distinct and modified or inducible in response to different substance.

Distribution in the Tissues

The fatty acid desaturation activity is not evenly distributed in all the organs. Dr. Catala in our laboratory prepared the microsomes of several tissues of young rats (45 days old) and measured the per cent conversion of linoleic acid to γ-linolenic acid for 5 mg of microsomal protein (Table III). The results indicated that only liver, testicles and adrenals gave measurable conversions in our experimental conditions compared to heart, epididymal fat, brain, lungs and kidneys. However, Gellhorn and Benjamin (19) found that adipose tissue was able to desaturate stearic to oleic acid.

These results may suggest that olefinases are not evenly distributed among all the organs and that heart and other organs may depend on the liver for the synthesis and supply of arachidonic acid. These hypotheses were coincidently suggested by Nervi and Brenner (20) in 1964, studying the comparative incorporation of arachidonic acid in liver and heart when fat deficient rats were fed with linoleate or arachidonate.

TABLE III

Linoleic Acid Desaturation to γ-linolenic Acid in Different Rat Tissues

Tissue	Conversion, %
Liver	13.9
Adrenals	19.8
Testicles	6.3
Heart	<1.1
Kidney	<1.6
Brain	<2.0
Lung	Not measurable
Epididymal fat	Not measurable

[a]Five milligrams of microsomal protein of the pool of 32 rats incubated 20 min at 35 C with 10 nmoles -1-^{14}C-linoleic acid in the conditions of experimental part.

TABLE IV

Testicular Desaturation of Linoleic to γ-Linolenic Acid (27)

Age, weeks	Conversion, %
3	30.0
5.5	16.0
6.0	15.3
6.5	11.0
18.3	7.0
25.5	1.8

[a]Four milligrams of microsomal protein incubated 20 min at 35 C with 10 nmoles -1-^{14}C-linoleic acid and cofactors.

Effect of Aging

The percent desaturation of linoleic acid to γ-linolenic acid decreases with the age of the animal. Younger animals desaturate more. This change has been especially studies in rat testicles (21) (Table IV). The very high desaturation found in the very young animals may be correlated to the building of new tissues, considering that polyunsaturated acids are very important components required for the synthesis of phospholipids, and the phospholipids associated to proteins are fundamental constituents of metabolically active membranes. The mechanism that controls the fatty acid desaturation activity in this case is not known. However, it may be probably correlated to the proteical effect described by Inkpen et al.(15) and Gomez Dumm et al.(16). Peluffo et al.(22) have attributed the activating effect of proteins on linoleic acid desaturation to a direct or indirect enzymatic induction.

Factors that Modify the Desaturation of Fatty Acids in the Microsomes

Some of these factors have been already shown in Table II and a general shceme has been already published (6). But before considering these factors it is necessary to emphasize that the desaturation of fatty acids is produced in the endoplasmic reticulum where other reactions also take place. These reactions are acylation of CoA, transesterification of fatty acids to synthesize the different classes of lipids, lipolysis and acyl-CoA hydrolysis. In consequence, these reactions may contribute to modify the picture obtained in experiments in vitro in which the desaturating enzyme is only considered. On the other hand, the conditions in vivo may be different to the conditions in vitro. The availability of substrate that may be gradually provided to the cell in contact with a

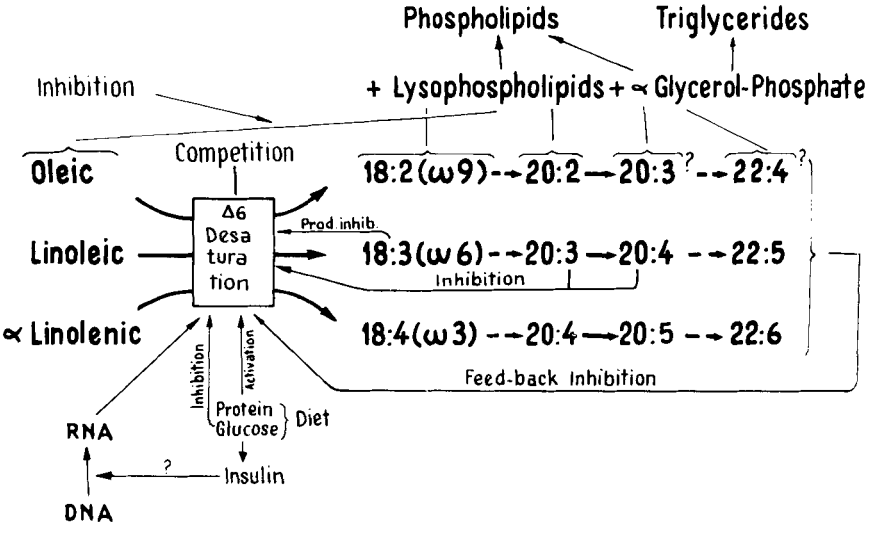

FIG. 4. Scheme of factors that may modify fatty acid 6-desaturation in microsomes.

TABLE V

Olefination of Fatty Acids: Oxidative Desaturation of *Cis* and *Trans* Acids[a]

Fatty acid		Position of new double bond	Conversion, %
	16:0	9-10	50.0
	18:0	9-10	32.1
Cis	18:1	6-7	6.0
Trans	18:1	-	0
Cis	18:2	6-7	13.7
All trans	18:2	-	0
α	18:3	6-7	39.7

[a]Measured for 5 mg microsomal protein of rat liver incubated 20 min in air with 10 nmoles 1-^{14}C acid and cofactors.

high enzyme concentration may distort completely the effect expected from experiments in vitro, in which relatively high amounts of substrate are added to a relatively low concentration of enzymes.

The factors that modify the yield of the desaturation may be summarized in two groups: (a) factors of immediate effect and (b) of delayed effect.

The factors of immediate effect evoke an instantaneous modification of the desaturation and may be classified in competitive reactions among fatty acids (a) of different series (2,6), (b) product inhibition (6,21), (c) feedback inhibition (23), and (d) inhibition evoked by intermediate members (21,24); also in competition of the desaturating enzyme with the transacylating enzymes for the fatty acids (25).

The factors of delayed effect provoke a modification after a few hours. They are attributed to the induction of enzymes, involved directly or indirectly with fatty acid desaturation. These factors may be dietary, as it has been shown in Table II for carbohydrates (17) and proteins (15,17,22) or hormonal. Insulin has been thoroughly studied in this respect (17,19,22,26,27). It enhances fatty acid desaturation, but it will not be discussed here. A general scheme of all these factors is represented in Figure 4.

A new factor has been recently found by Brenner and Catala (submitted for publication), which may modify the yield of 6-olefination of linoleic, α-linolenic and oleic acid, but not the 9-olefination of palmitic and stearic acid. This factor is ATP. Microsomes preincubated with ATP in N_2 at pH 7.0 enhance their desaturation capacity and when linoleic acid or linoleyl-CoA are added and incubated in the usual way with the appropriate cofactors, an approximate increase of 50% is found when compared to the nonpreincubated microsomes. This effect is specific of ATP. However, its physiological role is not yet well understood.

Hypothetical Model of 6-Olefinase

A careful comparison and evaluation of the data collected when studying the desaturation of unsaturated fatty acids with different number and position of double bonds, stereoisomeric forms as well as mutual competitive effects have led us to propose a model of 6-olefinase. In this model, the structural configuration of unsaturated fatty acids generally admitted, has been compared to their inhibitory effects. The structures were designed using Vandenheuvel's procedure (28). The possible binding forces that may collaborate to determine the position of the acyl-CoA on the endoplasmic reticulum has also been analyzed, as well as the modern theories of membrane structure.

The existence of a double bond between carbons 9-10 and the opening of a new double bond in divinyl position at carbons 6-7, are common features of oleic, linoleic and α-linolenic acid. Besides, the increase of the per cent desaturation (Table V) and mutual inhibition of fatty acids with the number of double bonds have been proved in experiments in vitro (2) or in vivo (29). These results have led us to consider that the 9-10 double bond, the hydrocarbon tail and the other double bonds in position 12-13 and 15-16 of these acids are recognized by the 6-olefinase. With these conclusions, we tentatively propose, in the first place, that the 6-desaturation of oleic, linoleic and α-linolenic acid would be produced by the same enzyme. Enough evidence has been provided to accept this proposition without much hesitation (Table II) (2). In the second place, it is suggested that the enzyme would have a structure able to recognize and bind the hydrocarbon tail and double bonds specified above in fixed positions, in such a way that the carbons 6-7 would be in front of the "desaturating site." The desaturating site would be connected with the microsomal electron transport chain that, in consequence, must be situated in its neighborhood.

The hypothesis then supposes that the binding occurs from double bond 9-10 to the tail of the unsaturated acid, that the binding of double bond 9-10 is essential, and that double bonds 12-13 and 15-16 would strengthen the binding.

Considering the tridimensional configuration of unsaturated fatty acids and with the help of Vandenheuvel's procedure (25), a tentative structure of the 6-olefinase is proposed, and has been designed by Actis Dato. In this design, the double bonds are considered coplanar and

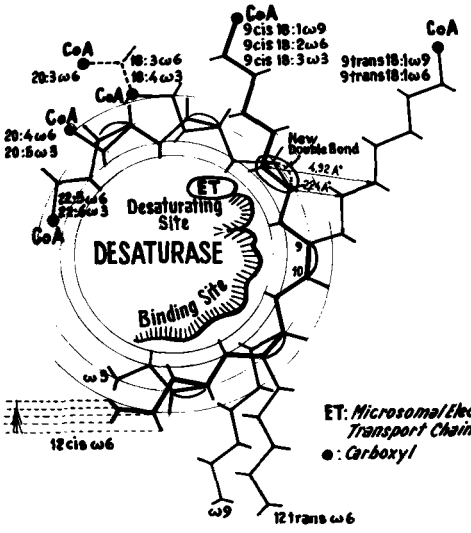

FIG. 5. Tentative model of binding and desaturating sites of 6-olefinase.

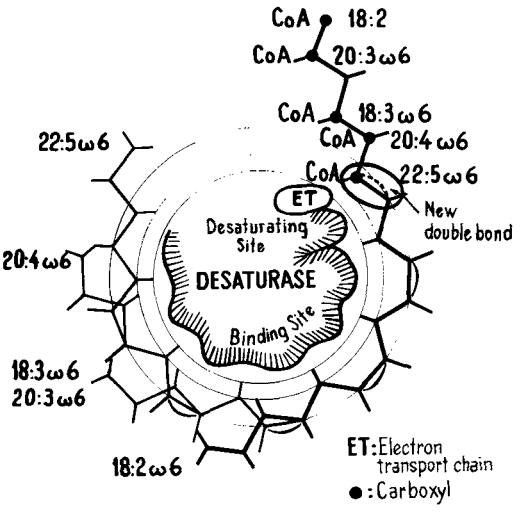

FIG. 6. Alternative possible binding of polyunsaturated acids of linoleic acid series to the 6-olefinase.

producing a pronounced bend of the molecule. However, this structure is not essential for the hypothesis, and a helical configuration that is considered to assume also polyunsaturated fatty acids may be even more suitable to bind to helical proteins. Therefore, what is essential in the hypothesis is that whatever the real structure of the unsaturated fatty acids may be, the configuration and binding forces of the enzyme must mimic the hydrophobic tail, from the 9-10 double bond and so on.

The hydrocarbon tail of the fatty acid is essentially hydrophobic. Therefore, London-Van der Waals dispersion forces would be able to provoke the binding. Since the disperson forces vary as $1/_D7$, being D the distance between the interacting molecules, the substrate and the lipoproteic enzyme must be close enough to make the binding effective. These forces increase with the number of carbon units of the molecule and in consequence they may be quite large. Salem (30) has calculated the effect of each $-CH_2-$ at an interaction distance of 5 A to provoke a London-Van der Waals dispersion energy of:

$$\frac{\text{W disp.}}{\text{N}} = 0.4 \text{ kilocal/mole}$$

being N equal to the number of identical units. This strengthening of the London-Van der Waals forces with the number of $-CH_2-$ may be applied to our hypothesis to explain the increased binding and in consequence, the increase of desaturation and inhibitory power from oleic to linoleic and α-linolenic acid. However, the double bonds must very probably exert not only a geometric effect due to the kink they provoke on the fatty acid chain, but an attracting effect due to their π electrons that are polarizable. There may be an overlap of π orbitals of the olefinic double bonds and of those of alternate N-C-0 π systems of a helical enzyme. It is convenient to remark that the polar effect of double bonds is used daily in gas liquid chromatography.

Whereas London-Van der Waals forces would be essential to bind the fatty acid to the enzyme, they have a short range. Therefore, long range forces may play a role in attracting the acyl-CoA enzyme. Electrostatic forces would be adequate to provoke this effect. Hence the -CO-S CoA, being the polar group of the acid, would very probably be responsible for the attraction. However, this is a suggestion and not a necessity of the model.

The model proposed in Figure 5 has been tested with other unsaturated fatty acids, of linoleic and α-linolenic acid families, as it is shown in the same Figure. The acids tested are γ-linolenic, arachidonic, eicosa-8,11,15-trienoic, docosa-4,7,10,13,16-pentaenoic, and docosa-4,7,10,13-16,19-hexaenoic. All the acids were experimentally shown to inhibit linoleic acid desaturation (21,23,24,31). The model fits quite well with these results when the acids are designed in the way considered in Figure 5, since they overlap in the desaturating site. However, the same results would be obtained with the model if the acids were fixed to the

enzyme in the way shown in Figure 6. That is, the double bond that is nearest to the carboxyl group would be recognized by the binding site that corresponds to carbons 9-10 of oleic acid. Which of the two situations is the most appropriate is difficult to elucidate with our actual experience.

The structure of Figure 6 would be nearly obligatory in the event that the 6-olefinase were the same enzyme that desaturates the unsaturated acids of 18, 20 and 22 carbons. However, this hypothesis has not yet been proved. The fact that linoleic acid depresses eicosa-8,11,14-trienoic acid desaturation (24) does not add further evidence in fabor of any of the two situations. Besides, Actis Dato and Brenner (31) have shown that while docosa-4,7,10,13,16-pentaenoyl-CoA is a stronger depressor for linoleyl-CoA desaturation, docosa-4,7,10,13,16,19-hexaenoyl-CoA is a stronger inhibitor than docosa-4,7,10,13,16-pentaenoyl-CoA for αlinolenyl-CoA desaturation. Both results fit again equally well in both schemes.

The general model has in addition another important advantage. It may differentiate *cis* from *trans* double bonds. In the model designed in Figure 5, it is proposed that the "desaturating site" would be at approximately 2.5 A from the orienting double bond binding site, and this orienting binding place may fix either *cis* double bonds or *trans* double bonds. If a *cis* acid is bound, it would be easily desaturated as is shown in Figure 5. However, if a *trans* double bond is fixed the desaturating site would be too far from the acid to desaturate the acid as is also shown in Figure 5. That is, our model predicts that: (a) elaidic acid will not be easily desaturated; (b) elaidic acid may produce a weak inhibition of linoleic acid desaturation, similar to that of oleic acid; (c) all *trans* linoleic acid cannot be easily desaturated; (d) 9 *cis*-12 *trans*-linoleic acid may be desaturated; (e) 9 *trans*-12 *cis*-linoleic acid cannot be easily desaturated; and (f) all *trans* linoleic acid may inhibit linoleic acid desaturation.

All these predictions agree with experimental results. The confirmation of (a) and (c) is shown in Table V. Experiments done in *vivo* by Privett et al. (32) confirm predictions (c)(d) and (e), whereas experiments of Selinger and Holman (33) confirm (c) and (f). Besides, our own experiments done in vitro (6) confirm predictions (b) and (f).

The structure attributed to the 6-olefinase also predicts that saturated acids would very probably produce very little or no inhibition of oleic, linoleic or α-linolenic desaturation. Experiments done by Brenner and Peluffo in 1966 (2) showed that equivalent amounts of stearic or palmitic acid did not inhibit significantly the desaturation of the aforementioned acids.

The desaturation of many unnatural unsaturated acids has been investigated and reported by several authors (34-38). However, these experiments have been done generally by feeding essential fatty acid defidient animals with the appropriate acid and studying the fatty acid composition of the tissues. In spite of their importance in the confirmation of the model of olefinase, these experiments may lead to erroneous interpretations, due to the many factors that act upon the whole animal. However, without analyzing them in detail, and in spite of the lack of the necessary studies of competition, they insinuate that our model must very probably also include a limitation of the space to be occupied by the fatty acid molecule.

The model admits further and necessary improvements and probably corrections to explain the challenging problem of unsaturated fatty acid olefination. It must include the interesting finding of Morris et al. (39) that the removal of the pair of hydrogen atoms is stereospecific and simultaneously concerted. This result was obtained in animal tissue for stearic acid desaturation and although it probably occurs in unsaturated acids, as far as we know it has not yet been proved.

One of the very important problems to be resolved is the determination of the identify of the binding site of the enzyme. We may speculate that the lipoproteic structure of the olefinase complex may be important. A lipoproteic structure of the type suggested by Benson (40) with the lipid molecules inserted in the basically hydrophobic protein network would fit rather well in the model proposed. However, to what extent the constitutive lipid moietes may also contribute to the binding forces of the enzyme is open to investigation.

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The Distribution of 17 Carbon Fatty Acids in the Liver of a Child With Propionicacidaemia

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ABSTRACT

A child with propionicacidaemia due to a defective propionyl CoA carboxylase activity, accumulated odd number fatty acids in his liver. Seventeen carbon saturated and monounsaturated acids both represented 2% to 3% of the total liver fatty acids. The monounsaturated acid is demonstrated to be heptadec-9-enoic acid. The distribution of the 17 carbon saturated and monounsaturated acids throughout the major lipid classes is described. Enzymic degradation of lecithins and triglycerides is used to establish the positional specificity of esterification of these two fatty acids. The results indicate that when the only difference between two fatty acids, present in similar concentrations, is a single double bond, highly specific esterification patterns are retained.

INTRODUCTION

Children born with a defective propionyl CoA carboxylase activity accumulate odd-numbered fatty acids (1,2). This is presumably due to propionyl CoA substituting for acetyl CoA as a primer in fatty acid synthesis (3). We have demonstrated that besides the 15 and 17 carbon saturated fatty acids present in the tissues of these children, there is also an appreciable amount of a 17 carbon monoenoic acid (2). It appears that the presence of similar amounts of 17 carbon saturated and monounsaturated fatty acids, each representing 2% to 3% of the total fatty acid pool, provides a unique opportunity to study the effects of both chain length and the presence of one double bond on the specificity of esterification of fatty acids in the liver in vivo. In this study, we have investigated the distribution of 17 carbon saturated and monounsaturated fatty acids in the major lipid classes present in the liver of the child in which we initially established the nature of the enzyme defect (2).

METHODS

A portion of liver was removed soon after death for enzyme assay (2), and a piece of this weighing 2 g was stored at -17 C. This material was thawed and extracted by the method of Folch et al. (4). The lipid extract was taken to dryness, dissolved in chloroform, filtered through glass wool and stored at -17 C under nitrogen. Aliquots of this chloroform solution were used for all subsequent analyses.

Neutral lipids were separated by thin layer chromatography on Silica Gel G using petroleum spirit (40-60 C)-diethyl ether-acetic acid (90:10:1 v/v). Phospholipids were also separated on Silica Gel G using chloroform-methanol-acetic acid-water (65:25:8:4 v/v) or chloroform-methanol-water (65:25:4 v/v). Lipids were visualized under uv light after spraying with 0.2% dichlorofluorescein in ethanol, and then were either eluted by the method of Arvidson (5) or transmethylated directly in 5% H_2SO_4 in methanol. Phospholipids were further identified by spraying thin layer chromatograms (TLC) with the modified Dragendorf reagent (6) or with 0.2% ninhydrin in acetone. Methyl esters were separated according to degree of unsaturation by $AgNO_3$ – silica TLC (7).

Analytical and preparative gas liquid chromatography (GLC) of methyl esters was performed on 15% DEGS on 100/120 mesh Diatomite C at 165 C on a Pye 104 Model 64 gas chromatograph. Dicarboxylic acids produced after periodate-permanganate oxidation of heptadecenoic acid (8) were analyzed as their methyl esters on the same column at 165 C; the methyl esters of the monocarboxylic acids produced were analyzed at 105 C. Fatty acid methyl esters were quantitated by multiplying peak height by retention time. The ratios of 17 carbon saturated and monounsaturated fatty acids in the various lipid fractions were established by measuring these two esters at high amplification to reduce the variability usually found in measuring minor constituents of complex mixtures.

Isolated samples of lecithin and phosphatidyl ethanolamine were digested with snake venom phospholipase A_2 (*Crotalus adamanteus*) (9) and the products were separated by TLC. Triglycerides were digested with porcine pancreatic lipase (10). The results of enzyme degradations were shown to be satisfactory by summing the fatty acid compositions of the products.

TABLE I

Fatty Acid Composition (%) of the Major Lipid Classes

Fatty acid	Total lipid	FFA[a]	Cholesterol esters	TG	PC	PE
14:0	1.2	1.2	4.1	1.8	0.3	0.7
15:0	1.2	0.85	2.95	1.7	0.9	0.3
16:0	34.3	17.7	24.7	38.2	32.7	13.9
16:1	5.9	9.6	8.1	6.8	2.1	1.2
17:0	3.1	1.6	2.6	3.1	2.4	3.25
17:1	2.35	4.0	2.9	3.1	1.6	1.0
18:0	14.7	6.4	7.3	6.3	11.3	23.7
18:1	22.3	32.9	28.6	31.1	16.0	5.0
18:2	3.95	6.2	3.4	3.3	5.6	2.35
20:4	8.1	14.1	12.0	2.4	24.0	39.5
Ratios						
$\frac{17:0}{17:1}$	1.3	0.40	0.90	1.0	1.5	3.2
$\frac{16:0}{18:1}$	1.5	0.54	0.86	1.26	2.0	2.8

[a]Abbreviations: FFA, free fatty acid; TG, triglyceride; PC, phosphatidyl choline; PE, phosphatidyl ethanolamine.

RESULTS

Identification of Heptadec-9-enoic Acid

This fatty acid was first identified as a heptadecenoic acid by plotting the log of the retention time against carbon number. The presence of this fatty acid in the monoene fraction isolated by silver nitrate TLC and the effect of catalytic hydrogenation, further established that it was a heptadecenoic acid. The methyl ester of this acid was isolated from the monoenoic fraction by preparative GLC and then subjected to periodate-permanganate oxidation. The mono- and dicarboxylic acid fragments were analyzed separately and shown to be almost exclusively octanoic, and azelaic acids respectively. This established the structure as heptadec-9-enoic acid.

Fatty Acid Composition of the Major Lipid Classes

Both the total fatty acid pattern and also the fatty acid composition of the major lipid classes are presented in Table I. The ratios between the amounts of heptadecanoic and heptadecenoic acids are given below each column; and the individual lipid classes are presented in ascending order of this ratio. The greatest degree of 17 carbon unsaturation is found in the free fatty acid fraction while phosphatidyl ethanolamine has the greatest proportion of saturated 17 carbon acids. An attempt was made to correlate the ratio of saturated to unsaturated 17 carbon acids with some other characteristic of the fatty acid pattern of each lipid class. The only parameter that appeared to be related in any way was the palmitic acid to oleic acid ratio, and this ratio is also presented in the Table. The overall pattern of the distribution of saturated, unsaturated and polyunsaturated fatty acids between each lipid class in no way appears abnormal except for the presence of odd-numbered acids.

Relationship Between Chain Length and Degree of Unsaturation in the Total Liver Fatty Acid Fraction

The ratio of 17 carbon-unsaturated to saturated fatty acids in the total saponified fatty acid fraction, i.e., the degree of desaturation of the total 17 carbon acyl pool, is compared with the equivalent desaturation ratios for palmitic and stearic acids in Figure 1. The extent of the desaturation of heptadecanoic acid is intermediate between palmitic and stearic acids, resulting in almost equal amounts of heptadecanoic and heptadecenoic acids available for esterification.

The Positional Distribution of 17 Carbon Fatty Acids in Lecithin

The lecithin fraction was isolated and degraded with snake venom phospholipase A2 (Table II). The fatty acids from the 1 position were predominantly saturated, i.e., 62% palmi-

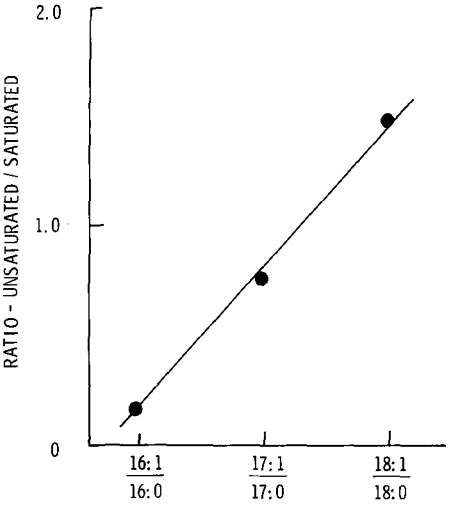

FIG. 1. The effect of chain length of the desaturation of 16, 17 and 18 carbon fatty acids. The ratio of monounsaturated to saturated fatty acid is plotted against the chain length of each pair. This data is calculated from the per cent composition of the total saponified fatty acid fraction.

tic, 20% stearic and 5% heptadecanoic acid. The fatty acids from the 2 position were highly unsaturated containing 42% arachidonic acid and 24% oleic acid. In the 1 position there was a great excess of heptadecanoic acid over heptadecenoic acid, 5 to 1, and this ratio was almost exactly reversed in the 2 position. Correlation between the 17 carbon saturated and unsaturated ratio and the palmitic acid to oleic acid ratio in each individual position was less than that for the whole molecule.

The Positional Distribution of 17 Carbon Acids in Triglyceride

The triglyceride fraction was degraded with pancreatic lipase and the resulting free fatty acids and monoglycerides analyzed (Table II). In view of the pathway of de novo synthesis of lecithins and triglycerides from a common diglyceride precursor, the fatty acid compositions of the 2 position of lecithin and triglycerides were compared to determine the degree of modification of the composition of lecithin by other contributing synthetic pathways. The composition of the 2 position of the triglyceride fraction differed appreciably from that of the 2position of the lecithins. The 2 position of the triglyceride fraction contained only 1.2% arachidonic acid compared with 42% in the same position in lecithin. Recalculation of the percentage composition of the 2 position of lecithin after exclusion of arachidonic acid shows that the massive increase in arachidonic acid is not the only difference between the 2 positions. In the triglyceride 2 position there are approximately equal amounts of 16 and 18 carbon acids while in the lecithin 2 position 18 carbon acids predominate.

There are also significant differences between the 17 carbon acid ratios between the 2 positions of triglyceride and lecithin. In triglyceride 2 position the 17 carbon acid saturated to unsaturated ratio is 0.37, indicating a less

TABLE II

Positional Distribution of Fatty Acids (%) in Lecithin and Triglycerides

Fatty acid	Lecithin		Triglyceride	
	1 Position	2 Position	2 Position	1+3 Position
14:0	0.4	0.9	1.6	2.3
15:0	1.5	0.8	1.7	2.1
16:0	61.9	10.7	30.8	40.7
16:1	1.5	3.7	10.3	4.4
17:0	5.1	0.46	1.46	3.6
17:1	1.0	2.2	3.95	2.6
18:0	19.7	3.3	2.8	11.0
18:1	5.2	24.1	35.6	26.2
18:2	0.6	6.8	5.8	2.2
20:4	0	42.2	1.2	3.7
Ratios				
$\frac{17:0}{17:1}$	5.1	0.21	0.37	1.38
$\frac{16:0}{18:1}$	11.9	0.44	0.87	1.6

specific selection of the monounsaturated acid than in the lecithin 2 position.

DISCUSSION

Heptadecanoic and heptadecenoic acids are not uniquely confined to the tissues of children with propionicacidaemia; many workers have reported fatty acid compositions containing up to 1% of heptadecanoic acid in mammalian tissues and occasionally a trace of heptadecenoic acid has been demonstrated. Kishimoto and Radin (11), for example, reported that heptadecenoic acid is present in pig brain at a concentration of 2.8 mg/100 g brain. The presence of 2-3% of both 17 carbon saturated and monounsaturated acids in the liver of this child, and similar amounts of these two acids in his red cells (2) in therefore greatly in excess of normal concentrations.

The almost exclusive localization of the double bond to the 9,10 position of heptadecenoic acid demonstrated in this study is in keeping with the known specificity of animal long chain fatty acid desaturases (12) and the relative degrees of desaturation of 16, 17 and 18 carbon acids in this liver (Fig. 1) compares well with the chain length specificity of mammalian desaturases found by Paulsrud et al (13). The difference between the relative proportions of palmitic and palmitoleic acids and the relative proportions of stearic and oleic acids in animal tissues makes it difficult to differentiate between the effects of chain length and the effects of a single double bond on esterification patterns. The desaturation ratio for heptadecanoic acid found here insures that similar amounts of saturated and unsaturated 17 carbon acids are available for esterification (Fig. 1).

The overall fatty acid composition of the major lipid classes (Table I) is similar to those described in the very large number of lipid analyses already in the literature. The general characteristics of each of these lipid classes are reflected in the relative proportions of heptadecanoic and heptadecenoic acids present. The free fatty acid fraction contains the largest amounts of monounsaturated acids (16:1 and 18:1) and this is reflected in the low 17:0/17:1 ratio. Phosphatidyl ethanolamine contains the highest content of stearic acid, and the lowest proportion of monounsaturated acids, and this is reflected in the highest 17:0/17:1 ratio. Although this general correlation exists, it appeared useful to try and identify in more detail the factors regulating the proportions of heptadecanoic and -enoic acids selected for each lipid class. In attempting to correlate the ratios of seventeen carbon acids in each lipid class with some factor related to the degree of unsaturation, the only parameter that seemed to be related was the palmitic acid to oleic acid ratio.

Each enzyme involved in fatty acid esterification demonstrates a chain length optimum for both the saturated and the monounsaturated series, and the pattern of the fatty acid composition of each lipid class is the resultant of the chain length specifities of several enzyme activities. The relationship between the saturated and unsaturated 17 carbon acids and the palmitic to oleic acid ratio must be an overall reflection of these specifities. A possible explanation for this particular relationship is that the extent of esterification of heptadecanoic acid corresponds more nearly to that of palmitic acid than to that of stearic acid and similarly heptadecenoic acid more nearly approaches oleic acid than palmitoleic in esterification pattern.

The degree of unsaturation has more effect than the chain length of a fatty acid on the distribution of fatty acids between the individual acylation positions on a glycerophosphatide. This is demonstrated by the preferential localization of saturated acids to the 1 position and unsaturated acids to the 2 position of lecithin and phosphatidyl ethanolamine (14). In the present study the change in saturated to unsaturated ratio of the 17 carbon fatty acids from 5 to 1 in the 1 position of lecithin to 1 to 5 in the 2 position is a striking demonstration of the effect of a single double bond on the specificity of the mechanisms involved in establishing the fatty acid distribution throughout the lecithin molecule.

One of the major pathways for the de novo synthesis of lecithin is from diglyceride and CDP choline. Comparison of the fatty acid composition of lecithin and diglyceride will indicate the contribution of other pathways in determining the fatty acid composition of lecithin (15). The diglyceride pool is also used directly for the synthesis of triglycerides and so in the absence of data about the composition of the diglyceride fraction, comparison of the fatty acids at the 2 positions of triglyceride and lecithin should help to indicate the contribution of other pathways in determining the fatty acid composition of lecithin. The fatty acids in the 2 position of lecithin isolated in this study show a striking difference in composition from the fatty acids in the 2 position of triglycerides; lecithin containing 42% arachidonic acid while triglyceride contains only 1.2%. The distribution of fatty acids in the 2 position of lecithin after exclusion of arachidonic acid also shows

other differences from the 2 position of the triglycerides. These differences may be due either to synthesis of lecithin from phosphatidyl ethanolamine (16,17) or by modification of the lecithin after synthesis by the acylation-deacylation cycle (18). Digestion of the phosphatidyl ethanolamine fraction by phospholipase A_2 showed that the 2 position fatty acids contain approximately 80% arachidonic acid and only trace amounts of the 17 carbon acids. This high proportion of arachidonic acid in phosphatidyl ethanolamine makes it possible that the arachidonyl species of lecithin could have been derived by the methylation of phosphatidyl ethanolamine (17). The low proportion of the species other than arachidonyl species in phosphatidyl ethanolamine probably insures that methylation would have little effect on the overall pattern of fatty acids in the 2 position of lecithin other than to increase the percentage of arachidonic acid. Differential rates of methylation of the arachidonyl and the minor species (17) could be invoked to explain the full changes in the fatty acid patterns between the 2 positions of triglycerides and lecithins, but the operation of the acylation-deacylation cycle (18) is a more likely explanation. The changes in the 17:0 to 17:1 ratio between the 2 positions of triglyceride (0.37) and lecithin (0.21) are compatible with the operation of this cycle; during the reacylation reactions at the 2 position 17:0 would tend to be excluded and the re-esterification of 17:1 favored.

This study of the distribution of 17 carbon fatty acids in the liver of a child with propionicacidaemia will complement those in vitro studies of the effect of chain length on desaturation and esterification reactions that have used odd-numbered fatty acids (13,19-21) and illustrates the value of using odd-numbered fatty acids in investigations of the factors determining the fatty acid composition of various lipid classes.

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Incorporation of Oxygen-18 Into the Oxirane Ring of *cis*-9,10-Epoxyoctadecanoic Acid[1]

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ABSTRACT

The synthesis of *cis*-9,10-epoxyoctadecanoic acid by tissue slices of wheat plants infected with *Puccinia graminis tritici* (Wheat stem rust) has been investigated further. Synthetic methyl *cis*-9,10-epoxyoctadecanoate and the same ester isolated from incubations in an atmosphere containing $^{18}O_2$, or a medium containing $H_2{}^{18}O$, were analyzed by mass spectrometry. These analyses revealed that molecular oxygen was incorporated into the oxirane ring of *cis*-9,10-epoxyoctadecanoic acid.

INTRODUCTION

In previous studies concerning the biosynthesis of *cis*-9,10-epoxyoctadecanoic acid by tissue slices of wheat plants infected with *Puccinia graminis tritici*, atmospheric oxygen or light was found to be required for the synthesis of this acid (1). Presumably, enough oxygen was liberated through photosynthesis to satisfy the oxygen requirement, since the rate of epoxy acid synthesis was essentially the same whether incubations were performed with a nitrogen atmosphere and light, or with air and darkness. The relationships between unsaturated fatty acid synthesis, light and oxygen were the same as that observed for epoxy acid biosynthesis.

Previous data indicated that oleic acid, or one of its derivatives, was an immediate precursor to the epoxy acid. Therefore one could not say whether the oxygen requirement for the synthesis of this acid was due to the oxygen requirement for the synthesis of oleic acid or for the conversion of oleic acid to the epoxy acid, or both. Furthermore, an oxygen requirement for the conversion of oleic acid to the epoxy acid would not establish the origin of the oxygen in the oxirane ring. The purpose of this study was to determine the origin of the oxygen in the oxirane ring of the *cis*-9,10-epoxyoctadecanoic acid biosynthesized by rust-infected wheat plants.

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MATERIALS AND METHODS

The source and preparation of the wheat plant tissue which was infected with *Puccinia graminis tritici* was the same as that reported previously (1).

In one experiment 1.5 g of washed tissue slices were placed in a 125 ml Erlenmeyer flask which was covered to exclude light. Then 30 ml of 0.1 M potassium phosphate buffer (pH 4.5) and 0.8 μmoles of acetic acid were added. Using a rubber stopper fitted with a stopcock, the flask was attached to a vacuum manifold and evacuated to approximately 20 mm Hg. Then nitrogen was bled in to restore atmospheric pressure. This flushing process was repeated 10 times to remove $^{16}O_2$. Finally, the flask was evacuated to 20 mm Hg and the seal of the glass bulb containing 100 ml of $^{18}O_2$ was broken while the bulb was connected to the manifold. After allowing the $^{18}O_2$ to diffuse for 5 min, nitrogen was used to restore atmospheric pressure. From the volume of the system, the partial pressure of the $^{18}O_2$ was calculated to be approximately 270 mm Hg at the beginning of the incubation. The closed flask was removed from the manifold and the incubation performed for 10 hr at 25 C, with agitation.

In the second experiment, 1.5 g of the washed tissue slices were placed in a 125 ml Erlenmeyer flask which was covered to exclude light. Then 5.0 ml of a 0.1 M potassium phosphate buffer (pH 4.5) which had been prepared using $H_2{}^{18}O$, was added. After the addition of 0.8 μmoles of acetic acid, the flask was fitted with a cotton plug and the incubation was performed at 25 C with agitation, for 10 hr.

Methyl *cis*-9,10-epoxyoctadecanoate was isolated as reported previously (1). The final step in the procedure involved thin layer chromatography (TLC). The developed plate was sprayed with a 0.2% solution of Rhodamine-6G in ethanol and viewed under an UV lamp. The absorbent corresponding to methyl *cis*-9,10-epoxyoctadecanoate was scraped from the plate and extracted three times with chloroform. The sample was rechromatographed in the same system. After extracting the methyl *cis*-9,10-epoxyoctadecanoate from the absorbent, the purity of the sample was checked by gas liquid

TABLE I

Mass Spectral Data of Methyl *Cis*-9,10-Epoxyoctadecanoate From Incubations of Infected Wheat Tissue With $^{18}O_2$ and $H_2{}^{18}O$

Fragment, m/e	Percentage of base peak	Probable structures	Intensity ratio[a]		
			$^{18}O_2$	$H_2{}^{18}O$	Synthetic ester
155	83	$CH_3\text{-}(CH_2)\text{-}_7CH\text{-}CH$, $CH = CH\text{-}(CH_2)\text{-}_5C(=O)OCH_3$	0.56	0.13	0.10
171	14	199 - CO , $CH_3\text{-}(CH_2)\text{-}_7CH\text{-}CH\text{-}CH \cdot 2H$	0.25	0.07	0.06
199	12	$CH\text{-}CH\text{-}(CH_2)\text{-}_7C(=O)OCH_3$	0.32	0.19	0.06
214	2.4	$H \cdot CH_2\text{-}CH\text{-}CH\text{-}(CH_2)\text{-}_7C(=O)OCH_3$	0.21	0.14	0.09
281	1.8	M - CH_3O	0.17	0.12	0.08
74	100	$H \cdot CH_2\text{-}C(=O)OCH_3$	0.02	0.07	0.03

[a]The intensity of the F + 2 peak divided by the intensity of the F peak where F is the mass of the fragment given in the first column.

chromatography (GLC). Only one component, which exhibited the same retention time as authentic methyl *cis*-9,10-epoxyoctadecanoate, was observed in the extracts. Finally the sample was transferred to a 1.8 x 20 mm glass tube, and after evaporating the solvent, analyzed by mass spectrometry. Approximately 30 μg, of synthetic *cis*-9,10-epoxyoctadecanoic acid were treated with diazomethane and then isolated and analyzed in the same manner as the samples obtained from the incubations.

Mass spectra were obtained with a Hitachi model RMU-60, double focusing instrument with an ionizing potential of 80 ev and a maximum temperature of 160 C for the inlet oven.

The GLC procedures and the synthesis of *cis*-9,10-epoxyoctadecanoic acid have been described (1).

The $^{18}O_2$ (90 atoms per cent) and $H^{18}O_2$ (20 atoms per cent) were obtained from International Chemical and Nuclear Corp., Irvine, California.

RESULTS AND DISCUSSION

The gross features of the mass spectra of the methyl *cis*-9,10-epoxyoctadecanoate which was isolated from the $^{18}O_2$ and $H_2{}^{18}O$ incubations and the synthetic esters were very similar to the spectrum reported by Ryhage and Stenhagen (2) except that a base peak of 74 m/e was observed instead of 155 m/e. However, the 155 m/e peak was intense (<83% of the base peak) in our spectra. A lower inlet pressure due to a smaller quantity of sample could possibly account for this difference. The relative intensity of certain fragments differed considerably between the two experimental samples, and the synthetic ester. For fragments believed to possess the oxygen of the oxirane ring, intensity ratios were calculated. For a particular fragment (F), the ratio was the intensity of the F + 2 peak divided by the intensity of the F peak. Only fragments which exhibited a fairly intense peak were considered so that the F + 2 peaks were large enough for reasonably accurate measurements. The molecular ion, M, (312 m/e) of methyl *cis*-9,10-epoxyoctadecanoate was readily apparent (approximately 0.5% of the base peak, 74 m/e), but was not used since the M + 2 peak was very weak.

The data in Table I show that the intensity of the F + 2 peak relative to the F peak was greater for the epoxy ester isolated from the incubation performed with ^{18}O than for the synthetic ester, in all fragments except 74 m/e. The intensity ratios for the epoxy ester from the $^{18}O_2$-incubation are about four to five times greater than the intensity ratios for the synthetic ester when the fairly intense peaks, 155, 171, and 199 m/e, are compared. For the low intensity peaks, 214 and 281 m/e, the values of the intensity ratios differ by a factor

of two to three. Since the intensity ratios for the synthetic ester exceed the ratios calculated on the basis of the natural abundance of isotopes, one can assume that fragments other than those containing oxygen contribute to the F and F + 2 peaks, and the contribution of such fragments is variable among the different peaks listed. Therefore, a variable difference between the intensity ratios for the epoxy ester from the $^{18}O_2$ incubation and the synthetic epoxy ester should be noted. Errors in the measurement of peak intensities, particularly for the less intense peaks, 214 and 281 m/e, could also cause variability.

From the data, the percentage of molecules which contained ^{18}O cannot be accurately calculated, but it is low, probably in the order of 0.5% to 5%. This result is not surprising, for the quantity of the epoxy acid present in the tissue prior to the incubation would certainly dilute the isotopically-labeled acid which was synthesized during the incubation. However, the incorporation of $^{18}O_2$ into the oxirane ring of *cis*-9,10-epoxyoctadecanoic acid obviously did occur.

The intensity ratios for the methyl *cis*-9,10-epoxyoctadecanoate isolated from the incubation performed with $H_2{}^{18}O$, were greater than those of the synthetic ester, for all the fragments given in Table I. The differences in the intensity ratios between these two samples are not nearly as pronounced as the differences observed between the epoxy ester isolated from the ^{18}O incubation and the synthetic ester. However, the water was only enriched with ^{18}O to the extent of about 20 atoms per cent. Whether the differences in the intensity ratios between the epoxy ester from the $H_2{}^{18}O$ incubation and the synthetic esters are highly significant is questionable. From a comparison of the intensity ratios of the 74 m/e fragment, it appears that the ^{18}O of the water was incorporated into the ester function. However, after its synthesis, the epoxy acid is esterified in the rust uredospore (3). Virtually all of the ^{18}O in the ester group should have been removed by transesterification except the ^{18}O which may have been present in the carbonyl group.

The finding that molecular oxygen is incorporated into the oxirane ring of *cis*-9,10-epoxyoctadecanoic acid, indicates that the enzyme capable of catalyzing the epoxidation of the appropriate derivative of oleic acid is probably similar, in many respects, to the epoxidase which is responsible for the formation of 2,3-oxidosqualene (4). This molecule has been shown to be an intermediate in the conversion of squalene to lanosterol (5-8). As Bloch has stated, the requirement for NADPH and oxygen in the epoxidation of squalene seems to place the epoxidase in the mixed oxidase class of enzymes (4). The fact that the role of 2,3-oxidosqualene as an intermediate was unknown until recently, and that an epoxy steroid appears to function as an intermediate in a hydroxylation reaction (9) suggests that many unsuspected oxygenase reactions may involve an epoxide intermediate. If this is the case, knowledge concerning the formation and metabolism of the oxirane ring will assume greater significance.

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Thermophilic Fungi: II. Fatty Acid Composition of Polar and Neutral Lipids of Thermophilic and Mesophilic Fungi

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ABSTRACT

The relative per cents of polar and neutral lipids and the fatty acid profile of the polar and neutral lipids of nine thermophilic and nine mesophilic fungi were examined and compared. The polar lipids of the thermophiles contained an average of 0.89 double bonds per mole fatty acid (unsaturation index, USI) and were considerably more saturated than the corresponding lipids of the mesophiles (average 1.32 USI). Within the thermophilic species the polar lipids were generally more saturated than the neutral lipids (average 0.95 USI) and in the mesophilic species the polar lipids were usually more saturated than the neutral lipids (average 1.14 USI). The mesophiles produced higher levels of 16:1, 18:2 and 18:3 fatty acids than the thermophiles and preferentially incorporated 16:1 and 16:2 into their polar lipids. The thermophiles produce higher levels of saturated fatty acids and 18:1 than the mesophiles and preferentially incorporated the saturated fatty acids into their polar lipids.

INTRODUCTION

Two basic hypotheses have been rendered which attempt to explain the ability of organisms to exist at elevated temperatures. The first, which may be termed the dynamic hypothesis, attributes survival at elevated temperature to the rapid resynthesis of damaged or destroyed cell constituents (1). The second, the stable component hypothesis, ascribes either the intrinsic stability of cellular constituents or the presence of protective agents as the prime factor imparting temperature tolerance (2). Although the dynamic hypothesis may appear attractive it suffers from several inherent deficiencies. It does not explain the inability of thermophilic organisms to grow at normal temperatures, and, perhaps more significantly, it fails to take into consideration the thermal denaturation of proteins which are needed for synthesis of cellular constituents.

A considerable body of evidence has been accumulated supporting the stable constituent hypothesis. The high thermal stability of crude enzyme preparations isolated from a variety of organisms has been reported by numerous workers (3-6). Work done with pure enzyme preparations has been less extensive but the data which is currently available supports the stable constituent hypothesis more than the dynamic hypothesis.

The unique character of thermophilic fungi to thrive at temperatures at which many organisms perish may be based in part on the thermostability and functional permeability of the membranes. It had previously been shown that the total fatty acids of the thermophiles were more saturated than the fatty acids of comparable mesophiles (7-9). These differences (7) were not great and did not seem to aid in explaining thermophily. However, if these differences reside in the membrane lipids they would be highly significant. Consequently the fatty acid composition of the polar and neutral lipids of nine thermophilic fungi and nine similar mesophilic fungi were examined.

EXPERIMENTAL PROCEDURES

The mesophiles were grown at 25 C and thermophiles at 45 C. The organisms were cultured and extracted as previously reported (7). Aliquots (25 mg or less) of the total lipid extracts were divided into neutral and polar lipid fractions by chromatography on silicic acid columns (ATF silicic acid, 100-200 mesh, Supelco, Inc.). The neutral lipids were obtained by elution of the column (1 x 6 cm) with 25 ml of $CHCl_3$ and the polar lipid with 25 ml of CH_3OH. The fractions were evaporated to dryness under nitrogen in tared flasks, weighed, redissolved in $CHCl_3/CH_3OH$ (1:1) and stored at -25 C.

The methyl esters were prepared by transesterification of the lipids with 12.5% BF_3 in

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CH_3OH (10) and quantitated with a Microtek MT 200 gas chromatograph equipped with a flame ionization detector. Samples were chromatographed on a 6 ft 15% DEGS column at 185 C and peak areas were computed with an Aerograph 471 digital integrator.

RESULTS AND DISCUSSION

The percentage composition of polar and neutral lipids are shown in Table I. The polar lipid varied between 10.5% and 67.1%. There was no correlation between the composition found in the thermophiles and that found in the mesophiles. The fatty acid profile of the polar and neutral lipids of the 18 fungi examined are presented in Table II. In the mesophiles the concentrations of 18:0 and 18:1 were substantially higher in the neutral fractions than in the polar lipid fractions. The only exception to this generality was *Penicillium chrysogenum* where the polar fraction contained a slightly higher level of 18:1. With 18:2 the distribution pattern was substantially different. The polar fractions tended to contain higher levels of this acid, and only in *Chaetomium globosum* and *Humicola nigrescens* were there any substantial reversals in this pattern. The low 18:2 levels in the polar fractions of these two organisms is paralleled by an increase in the concentrations of 16:0, 16:1 and 16:2. The distribution of the other fatty acids between neutral and polar fractions appears to be entirely species dependent.

The distribution of fatty acids between the neutral and polar lipids of the thermophiles presents a different pattern from that observed for the mesophiles. The distribution of 16:0, 18:0 and 18:1 between fractions does not seem to follow any definite pattern. However, with 16:1 and 18:2 there appears to be a tendency against incorporation into the polar lipids the exceptions being *Chaetomium thermophile* and *Stilbella thermophila*.

The two temperature varieties demonstrate a distinct behavior in the synthesis and utilization of unsaturated fatty acids. The mesophiles produce higher levels of 16:1 and 18:2 than the thermophiles and preferentially incorporate these fatty acids into their polar lipids. Also the mesophiles produce significant amounts of 18:3 and incorporate it into the polar lipids to varying degrees. The thermophiles produce high levels of saturated fatty acids and 18:1. The saturated fatty acids are preferentially incorporated into the polar lipids. These factors combine to make the polar lipids of the thermophiles much more saturated than the polar lipids of the mesophiles.

The differences between the mesophiles and thermophiles are more readily apparent when

TABLE I

Percentage Polar and Neutral Lipids

Thermophile	Per cent neutral lipid	Per cent polar lipid	Mesophile	Per cent neutral lipid	Per cent polar lipid
Chaetomium thermophile	68.2	31.2	*Chaetomium globosum*	Not analyzed	Not analyzed
Humicola grisea var. *thermoidea*	81.2	18.8	*Humicola grisea*	59.5	40.5
Humicola insolens	32.9	67.1	*Humicola nigrescens*	79.5	20.5
Humicola lanuginosa	75.8	24.2	*Humicola brevis*	42.8	57.2
Malbranchea pulchella var. *sulfurea*	89.5	10.5	*Malbranchea pulchella*	83.6	16.4
Mucor pusillus	80.3	19.7	*Mucor globosus*	82.7	17.3
Penicillium duponti[a]	Not analyzed	Not analyzed	*Penicillium chrysogenum*	55.6	44.4
Sporotrichum thermophile	35.9	64.1	*Sporotrichum exile*	Not analyzed	Not analyzed
Stilbella thermophila	Not analyzed	Not analyzed	*Stibella* sp.	65.2	34.8

[a]Imperfect stage of *Talaromyces thermophilus*.

TABLE II

Fatty Acid Profile of Neutral and Polar Lipids

Organism	Lipid Fraction	14:0	15:0	16:0	16:1	16:2	18:0	18:1	18:2	18:3	20:0
Thermophiles											
Chaetomium thermophile	N[a]	1.4	2.0	46.2	5.5	2.0	3.4	10.2	29.3	---	---
	P	---	---	47.6	4.1	---	2.4	9.7	36.2	---	---
Humicola grisea	N	---	---	28.0	---	---	3.5	26.2	42.2	---	---
var. *thermoidea*	P	---	---	38.8	---	---	5.9	21.1	34.2	---	---
Humicola insolens	N	---	---	21.8	2.1	---	3.6	39.7	31.2	1.5	---
	P	---	---	27.3	1.6	---	1.2	37.7	29.9	1.8	---
Humicola lanuginosa	N	---	---	17.1	---	---	7.2	56.4	19.3	---	---
	P	---	---	26.5	---	---	8.7	48.8	14.6	1.3	---
Malbranchea pulchella	N	---	---	25.3	1.5	---	6.5	28.3	38.3	---	---
var. *sulfurea*	P	---	---	21.4	1.6	---	9.6	35.2	29.3	---	---
Mucor pusillus	N	---	---	26.7	3.2	---	3.1	48.1	16.4	1.5[b]	---
	P	---	---	24.5	1.4	---	---	55.6	16.4	1.4[b]	---
Penicillium duponti	N	---	---	22.3	---	---	12.1	40.8	24.3	---	---
	P	---	---	30.9	---	---	13.3	37.2	18.2	---	---
Sporotrichum thermophile	N	---	---	21.3	---	---	8.2	30.5	40.0	---	---
	P	---	---	30.1	---	---	6.3	31.6	32.0	---	---
Stilbella thermophila	N	2.9	---	36.3	3.1	---	12.9	28.8	16.0	---	---
	P	5.3	---	31.2	1.6	---	5.8	32.0	24.1	---	---
Mesophiles											
Chaetomium globosum	N	---	---	25.9	7.6	---	7.8	15.1	43.6	---	---
	P	---	---	26.3	16.1	2.1	2.8	11.5	41.3	---	---
Humicola grisea	N	---	---	19.4	---	---	3.5	29.4	38.1	9.5	---
	P	---	---	16.4	---	---	---	20.1	45.0	18.4	---
Humicola brevis	N	---	---	35.0	5.1	---	8.6	35.3	15.9	---	---
	P	---	---	31.2	4.2	---	2.4	25.4	35.2	3.8	---
Humicola nigrescens	N	---	---	18.8	1.7	---	9.2	36.4	25.8	8.0	---
	P	---	---	29.7	5.4	2.9	7.3	27.8	18.5	8.3	---
Malbranchea pulchella	N	---	---	11.5	---	---	15.9	30.6	41.9	---	---
	P	---	---	9.7	---	---	11.4	27.6	51.2	---	---
Mucor globosus[d]	N	5.8	---	30.3	1.7	---	7.3	36.1	6.5	12.3[c]	---
	P	3.9	---	17.4	5.3	---	3.5	31.4	12.8	25.7[c]	---
Penicillium chrysogenum	N	---	---	12.8	0.8	---	4.9	13.5	60.9	7.1	---
	P	---	---	13.4	1.8	---	3.8	14.1	60.4	6.5	---
Sporotrichum exile	N	---	---	24.2	4.6	4.7	10.2	16.9	34.8	1.9	2.7
	P	5.2	---	13.8	3.9	---	3.7	10.9	59.8	---	2.7
Stibella sp.	N	---	---	18.4	1.6	---	3.0	27.0	45.6	4.4	---
	P	---	---	31.8	2.3	---	---	11.3	51.6	3.0	---

[a]Abbreviations: N, neutral fraction; P, polar fraction.
[b]Mixture of γ-18:3 and 20:0.
[c]Hydrogenation data indicates that these peaks are almost entirely γ-18:3.
[d]Grown at 20 C.

TABLE III

Unsaturation Indices of Neutral and Polar Lipid Fractions

	USI[a] neutral	USI polar	USI Polar USI Neutral		USI neutral	USI polar	USI Polar USI Neutral
Thermophiles				Mesophiles			
Chaetomium thermophile	0.78	0.86	1.10	*Chaetomium globosum*	1.10	1.26	1.14
Malbranchea pulchella var.*sulfurea*	1.06	0.95	0.90	*Malbranchea pulchella*	1.14	1.30	1.14
Mucor pusillus	0.88	0.94	1.07	*Mucor globosus*	0.87	1.39	1.60
Humicola grisea var. *thermoidea*	1.11	0.89	0.80	*Humicola grisea*	1.34	1.61	1.20
Humicola insolens	1.09	1.04	0.95	*Humicola brevis*	0.72	1.11	1.54
Humicola lanuginosa	0.95	0.82	0.86	*Humicola nigrescens*	1.14	1.01	0.88
Penicillium duponti	0.90	0.75	0.82	*Penicillium chrysogenum*	1.57	1.56	0.99
Sporotrichum thermophile	1.10	0.95	0.86	*Sporotrichum exile*	1.06	1.34	1.26
Stibella thermophila	0.64	0.82	1.28	*Stibella* sp.	1.33	1.26	0.95
Average	0.95	0.89	0.96	Average	1.14	1.32	1.19

[a]Unsaturation index, double bonds per mole fatty acid.

the unsaturation index (USI, double bonds per mole) is considered, Table III. The neutral lipids of the thermophiles are more saturated (average 0.95 USI) than the mesophiles (average 1.14 USI). However, when the polar fractions are compared there is a more significant difference, for the thermophiles (average 0.89 USI) exhibit considerable lower levels of unsaturation than the corresponding mesophiles (average 1.32 USI).

The ratio USI polar lipids/USI neutral lipids is indicative of the relative prevalence of unsaturated fatty acids in the two fractions. When this value is greater than one the polar fraction is more unsaturated than the neutral fraction. In most of the thermophiles this value is less than one, indicating that there is discrimination against incorporation of unsaturated fatty acids into the polar lipids. The mesophiles generally demonstrated a tendency for greater incorporation of unsaturated fatty acids into the polar fraction.

Because the membranes must be in a liquid-crystalline state for growth (11) the more saturated nature of the polar lipids of the thermophiles provides greater thermostability and determines the lower temperature limit of functionality of the membranes. Perhaps the thermophiles cannot produce unsaturated fatty acids in sufficient quantity to maintain the membrane in a liquid-crystalline state at low temperature, and consequently, cannot grow at 25 C.

ACKNOWLEDGMENT

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Thermophilic Fungi: III. The Lipids of *Humicola grisea* var. *thermoidea*

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ABSTRACT

The lipids of the thermophilic fungus *Humicola grisea* var. *thermoidea* were qualitatively and quantitatively determined. The polar lipids consisted of 38.4-42.3% of the total lipids. The relative per cent phospholipids based upon the total phospholipids were as follows: phosphatidyl choline, 32.3-33.7%; phosphatidic acid, 24.5-31.7%; phosphatidyl ethanolamine, 15.8-20.9%; phosphatidyl inositol, 12.5-13.0%; phosphatidyl serine, 2.3-5.4%; and diphosphatidyl glycerol, 3.9-4.0%. The relatively high concentration of phosphatidic acid may be characteristic of fungi grown at elevated temperatures. Several sterol glycosides (3.1-6.0%) were present in the polar lipids. The neutral lipids consist of triglycerides, 28.6-36.0%; free fatty acids, 5.3-13.5%; sterols, 11.4-13.9%; sterol esters, 1.8-3.0%; and diglycerides, 2.2-3.4%. The sterols and derivatives comprise an unusually large fraction of the total lipids (16.3-22.9%) suggesting a role in thermostability.

INTRODUCTION

The fatty acids of the total lipids and in particular the polar lipids of thermophilic fungi, including *Humicola grisea* var. *thermoidea* have been shown to be more saturated than lipids in similar mesophilic species (1-4). The stability of the membranes at the elevated temperatures is undoubtedly due in part to the highly saturated nature of the fatty acids. This stability may also be partly due to other chemical constituents, proteins, sterols, polar lipids and their interactions. Since there has not been any detailed study of the lipid composition and specifically the polar lipids of any thermophilic fungi we now report such a study with *Humicola grisea* var. *thermoidea.*

EXPERIMENTAL PROCEDURES

Each Erlenmeyer flask (250 ml), containing 50 ml of medium (1,2,5), was inoculated with a 6 mm agarmycelium disc cut from a petri dish culture of *Humicola grisea* var. *thermoidea.* Cultures were incubated at 45 C with shaking (100 cpm; 4 in. path length) for 12 days. When radiochemically labeled lipids were desired, cultures were grown on a medium containing either 50 μCi of uniformly labeled ^{14}C-glucose, 0.5 mCi of $^{32}PO_4^{\equiv}$ or 1.0 mCi of $^{35}SO_4^{\equiv}$ for three to four days.

Mycelia from three culture flasks were collected, combined and washed with 150 ml H_2O on a tared fiberglass mat, lyophilized and weighed. The mycelial mat was placed in a homogenizing flask with 25 ml of $CHCl_3/CH_3OH$ (2:1 v/v) and homogenized for 3 min in an ice bath with a Virtis homogenizer. The mixture was filtered and the filtrate washed according to Folch et al. (6). The lipid extract was evaporated to dryness under nitrogen, redissolved in a minimal volume of $CHCl_3/CH_3OH$ (1:1 v/v), and stored at -25 C.

All thin layer analyses were performed on glass plates coated with a 0.5 mm layer of Brinkman Silica Gel HR. Polar lipids were analyzed by a two-dimensional system involving sequential development in the following solvents: A, chloroform-methanol-water-28% ammonia (130:70:8:0.5 by volume); B, chloroform-acetone-acetic acid-water

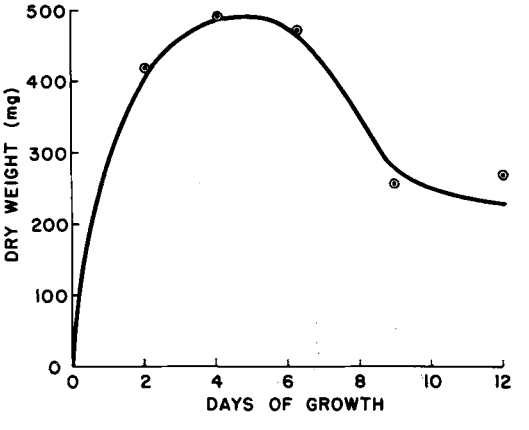

FIG. 1. Growth curve for *H. grisea* var. *thermoidea*. Weights were determined after lyophilization of the mycelia and each value is the combined weight from three flasks.

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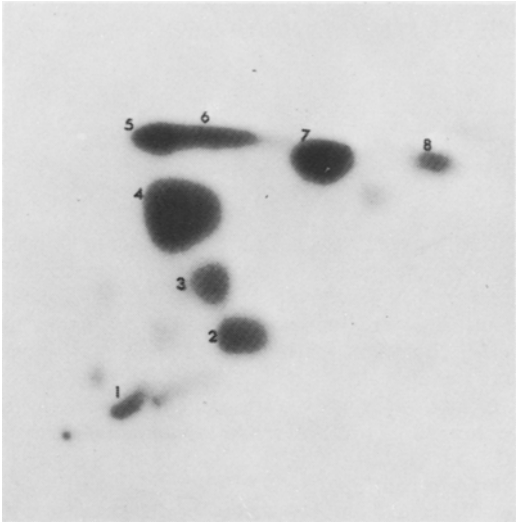

FIG. 2. Radioautogram of a thin layer chromatogram of the ^{32}P-labeled lipids of *H. grisea* var. *thermoidea*. The plate was developed in the x-direction with chloroform-methanol-water-28% ammonia (130:70:8:0.5 v/v) and in the y-direction with chloroform-acetone-acetic acid-methanol-water (100:70:20:20:10 v/v).

(100:40:20:10 by volume) (7). Tanks were fitted with paper liners. The neutral lipids were separated in a one-dimensional double development system. Plates were developed for two thirds of their length with ethyl ether-petroleum ether (30-60 C)-acetic acid (50:50:1 v/v/v), and were dried and developed for their entire length with ethyl ether-hexane (6:94 v/v). Also the Freeman-West solvent system was used (8).

Both neutral and polar lipids were visualized by radioautography, exposure to iodine vapor, charring with 20% H_2SO_4 and by *p*-toluenesulfonic acid spray for steroids (9). Lipids were tentatively identified by comparison of the R_f's of the unknown lipids to the R_f's of standard lipids (Supelco, Inc.). The identity of the polar lipids was further substantiated through deacylation experiments and by the utilization of the following sprays: Dragendorff (9), ninhydrin (9), Schiff's (10), Vaskovsky and Kostetsky (11), and pyrogallol (12).

The ^{32}P-labeled total lipids and the individual ^{32}P-labeled lipids, obtained by preparative thin layer chromatography (TLC), were deacylated by the method of Dawson (13). The deacylated products were assayed by two-dimensional paper chromatography on Whatman No. 4 filter paper. Chromatograms were developed with phenol-water (100:4 w/w) in the first direction and with 1-butanol-propionic acid-water (100:50:70 v/v/v) in the second dimension. Identity of the deacylated products was established by comparison of R_f's with those of deacylated ^{32}P-labeled *Chlorella* lipids (14).

The lipids were quantitated radiochemically. ^{14}C-lipids, from *H. grisea* var. *thermoidea* grown on uniformly labeled ^{14}C-glucose, were separated by TLC. The radioautograms thus obtained were used as a template for the removal of the individual lipid spots. The lipids, including the absorbent, were removed, placed in scintillation vials and were counted with a Tri-Carb liquid scintillation spectrometer (Model 526 Packard Instrument Co.). No significant quenching was observed when this technique was applied to ^{14}C-benzoic acid standards.

RESULTS AND DISCUSSION

Figure 1 shows a growth curve for *H. grisea* var. *thermoidea* grown under shake culture conditions. After the first three days of incubation the mycelium had developed as white globular pellets. By the fourth day a dark pigment appeared in the mycelium and intensi-

TABLE I

Reaction of Phosphatides to Various Spray Reagents

Spot[a]	Vaskovsky and Kostetsky	Ninhydrin	Schiff's	Dragendorff	I_2	H_2SO_4
1	+[b]	-	-	+	+	+
2	+	-	+	-	+	+
3	+	+	-	-	+	+
4	+	-	-	+	+	+
5	+	-	-	-	+	+
6	+	-	-	-	+	+
7	+	+	-	+	+	+
8	+	-	-	-	+	+

[a] ^{32}P- spots from radioautogram shown in Figure 2.
[b] +, Positive; -, Negative.

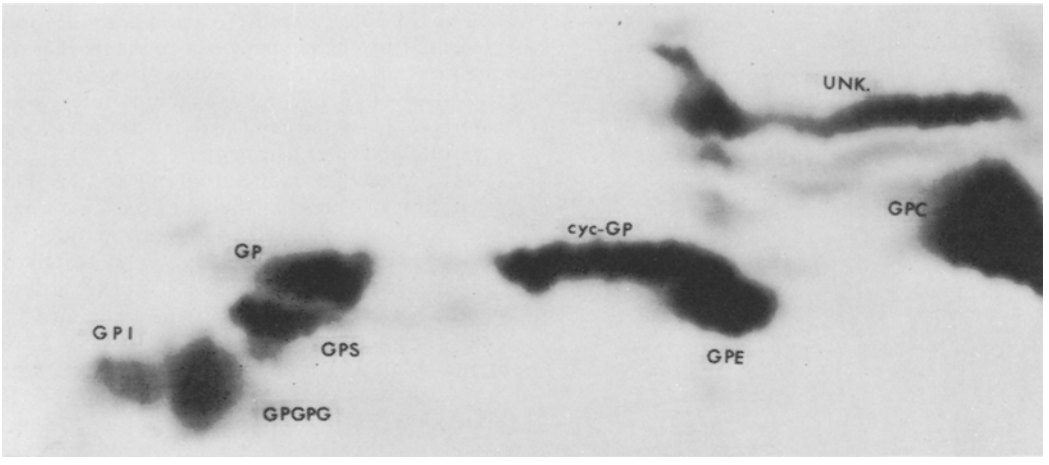

FIG. 3. Radioautogram of a paper chromatogram of the deacylated ^{32}P-lipids of *H. grisea* var. *thermoidea*. The paper chromatograph was developed in the x-direction with phenol-water (100:40, w/w), and the y-direction with 1-butanol-propionic acid-water (100:50:70 v/v).

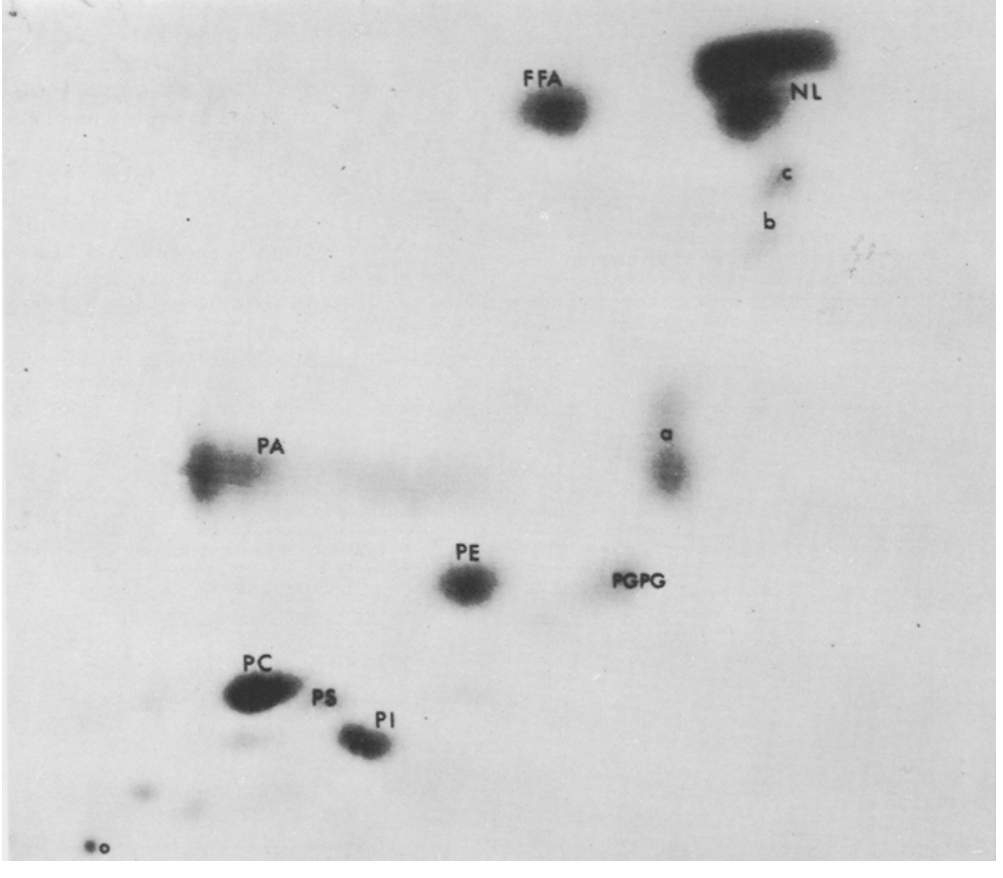

FIG. 4. Radioautogram of a thin layer chromatogram of the polar ^{14}C-labeled lipids of *H. grisea* var. *thermoidea*. The plate was developed in the x-direction with chloroform-methanol-water-28% ammonia (130:70:8:0.5 v/v) and with chloroform-acetone-acetic acid-methanol: water (100:70:20:20:10 v/v) in the y-direction.

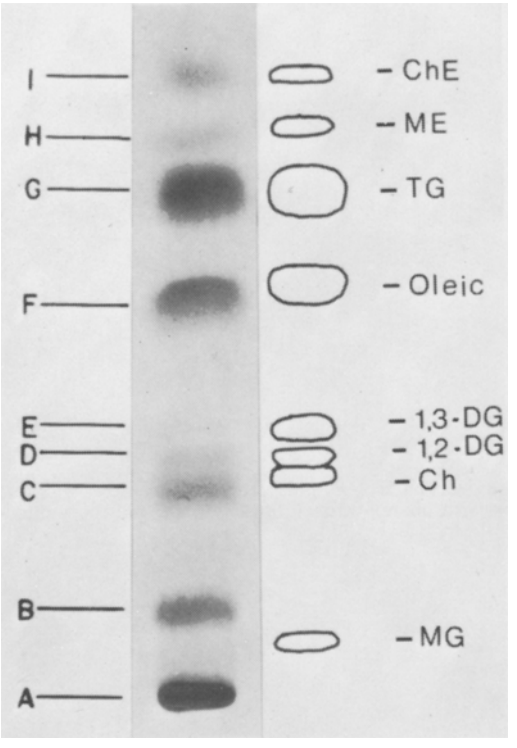

FIG. 5. Radioautogram of a thin layer chromatogram of the neutral ^{14}C-labeled lipids of *H. grisea* var. *thermoidea*. The plate was first developed for two thirds of its length with ethyl ether-petroleum ether (30-60 C)-acetic acid (50:50:1 v/v), followed by development for its entire length with hexane-ethyl ether (94:6 v/v). The right column shows the movement of standards: monoglyceride (MG), cholesterol (Ch), 1,2-diglyceride (1,2-DG), 1,3-diglyceride (1,3-DG), oleic acid, triglyceride (TG), fatty acid methyl ester (ME) and cholesterol ester (ChE).

fied as the culture aged. To avoid complications presented by the pigment and to insure that the quantitation was conducted with lipids representative of living mycelium, cultures were harvested between the third and fourth day in the radiolabeled experiments.

In Figure 2 a radioautograph of the TLC separation of the ^{32}P-labeled extract is shown. The R_f's of spots 2,3,4,7 corresponded to values obtained respectively with standards (Supelco Inc.) phosphatidyl inositol (PI), phosphatidyl serine (PS), phosphatidyl choline (PC) and phosphatidyl ethanolamine (PE). These spots exhibited the appropriate functional group analysis with spray reagents (Table I). Spots 5, 6 and 8 did not indicate the presence of any particular functional group, however, their relative positions suggested they might be phosphatidic acid (PA) and diphosphatidyl glycerol (PGPG). The total ^{32}P-lipids and the individual ^{32}P-lipids, isolated by preparative TLC, were deacylated by the method of Dawson (13). The deacylated products were analyzed by two-dimensional paper chromatography and compared to standards derived from ^{32}P-*Chlorella* lipids (Fig. 3). On the basis of these data the phospholipid spots in Figure 2 are as follows: 1, lyso-phosphatidyl choline; 2, PI; 3, PS; 4, PC; 5 and 6, PA (appears at both places); 7, PE plus a small amount of an unknown (determined by deacylation); 8, PGPG. No phosphatidyl glycerol was observed. No significant concentration of sulfolipids were detected when the culture was grown in the presence of $^{35}SO_4^=$.

When the ^{14}C-labeled extracts were separated by TLC in the polar solvent system several spots were observed in addition to the phospha-

TABLE II

Quantitative Composition of the Polar Lipid Fraction

	Relative per cent phospholipids		Per cent of total lipid	
Spot[a]	Sample[b]	Sample[c]	Sample[b]	Sample 2[c]
PA[d]	31.7	24.5	9.4 ± 0.5	9.5 ± 0.02
PE[e]	15.8	20.9	4.7 ± 0.1	8.1 ± 0.2
PGPG	4.0	3.9	1.2 ± 0.1	1.5 ± 0.01
PC	33.7	32.3	10.0 ± 0.3	12.5 ± 0.3
PS	2.3	5.4	0.7 ± 0.2	2.1 ± 0.2
PI	12.5	13.0	3.7 ± 0.3	5.0 ± 0.5
FA			14.2 ± 0.3	4.8 ± 0.1
NL			49.8 ± 0.6	52.9 ± 0.5
a			4.7 ± 0.2	2.1 ± 0.1
b + c			1.3 ± 0.1	1.0 ± 0.1

[a]The spot designation used here is the same as that used in Figure 4.
[b]Three-day incubation.
[c]Incubation of 3½ days.
[d]Abbreviations: PA, phosphatidic acid; PE, phosphatidyl ethanolamine; PGPG, diphosphatidyl glycerol; PC, phosphatidyl choline; PS, phosphatidyl serine; PI, phosphatidyl inositol; FA, fatty acid; NL, neutral lipid.

TABLE III

Quantitative Composition of the Neutral Lipid Fraction

Spot[a]	Identity	Percent of total lipid	
		Sample 1[b]	Sample 2[c]
A	Polar lipids	38.4 ± 0.8[d]	42.3 ± 0.9[d]
B	Sterol ?	10.4 ± 0.2	5.9 ± 0.1
C	Sterol	3.5 ± 0.1	5.5 ± 0.2
D	1,2-Diglyceride	1.5 ± 0.1	2.5 ± 0.3
E	1,3-Diglyceride	0.7 ± 0.05	0.9 ± 0.08
F	Free fatty acids	13.5 ± 0.5	5.3 ± 0.2
G	Triglycerides	28.6 ± 0.7	36.0 ± 1.0
H	Sterol ester ?	1.5 ± 0.07	Trace
I	Sterol ester	1.5 ± 0.01	1.8 ± 0.1

[a]Spots correspond to those depicted in Figure 5.
[b]Three-day incubation.;
[c]Incubation of 3½ days.
[d]These percentages are somewhat different from values reported for *Humicola grisea* var. *thermoidea* grown in stationary culture for four days (2).

tides (Fig. 4). Spots a, b and c gave positive reactions with Schiff's reagent and with *p*-toluenesulfonic acid suggesting they probably were sterol glycosides. The spots designated by FFA and NL are composed of free fatty acids and neutral lipids respectively.

Figure 5 shows the separation of the neutral lipids and their comparison to standard neutral lipids. Spots A, B, C, H and I gave positive reactions with the *p*-toluenesulfonic acid spray suggesting that they possessed some component which was steroidal in nature. On the basis of the various spray reagents, the behavior to alkaline and acid hydrolysis and the comparison to standards, the neutral lipids are identified as follows: A, polar lipids; B, unknown (probably a polar sterol); C, sterol; D, 1,2-diglyceride; E, 1,3-diglyceride; F, fatty acid; G, triglyceride; H, unknown (probably a polar sterol ester); I, sterol ester.

Tables II and III show the relative percentage of polar and neutral lipids. While the quantification procedures were highly reproducible within each experiment, statistically significant differences existed between the cultures incubated for 3 and 3 1/2 days. These differences are most pronounced in the concentrations of the free fatty acids, triglycerides and the proposed polar sterol in the neutral lipids, and with PE, and PS in the polar lipids. The

TABLE IV

Temperature Influence on Percentages of Phosphatides in Fungi

	Pythium[a] *ultimum,*		*Tricholma*[c] *nudum,*	*Candida*[d] *lipolytica,*		*Candida*[d] *scottii,*	*Humicola grisea* var. *thermoidea,*		*Agaricus*[g] *bisporus,*
Incubation temperature	20 C	30 C	room temperature	10 C	25 C	10 C	45 C[e]	45 C[f]	24 C
LPC[b]	3.0	6.2	---[h]	---	---	---	Trace[i]	Trace[i]	0.0
PA	6.2	10.7	7.0	h	h	h	31.7	24.5	0.0
PC	40.8	33.7	59.0	36	41	44	33.7	32.3	47.8
PE	22.2	24.1	20.0	29	20	24	15.8	20.9	50.2
PI	10.1	10.7	---	14	18	9	12.5	13.0	<1.0
PG	15.2	11.6	---	---	---	---	0.0	0.0	0.0
PGPG	1.7	1.8	---	<1	<1	4	4.0	3.9	0.0
PS	0.0	0.0	8.0	17	15	17	2.3	5.4	<1.0

[a]Incubated in stationary culture for eight days. Percentages were based on ^{32}P activity of components (16).
[b]Abbreviations: LPC, lyso-phosphatidyl choline; PG, phosphatidyl glycerol; also, per Table II.
[c]Grown in shake culture for four days. Percentages were based on fatty acid yields on saponification (17).
[d]Grown in shake culture for 65-70 hr. Percentages were based on the phosphorus content of the individual spots (15).
[e]Data calculated for the three-day-old culture.
[f]Data calculated for the 3½-day-old culture.
[g](18).
[h]Not reported.
[i]Present but not quantitated.

actual significance of these differences is difficult to interpret, however, it is conceivable that the variations observed reflect the increased utilization of free fatty acids by the aging culture for the synthesis of the phosphatides (PE and PS) and the triglycerides. The possibility exists that the high levels of free fatty acids in the three-day-old culture was the result of degradation of the phospholipids and triglycerides, however, this seems unlikely since there is no significant increase in lyso-phosphatides or diglycerides.

The phosphatides of *H. grisea* var. *thermoidea* are qualitatively similar to those reported by other workers for Deuteromycetes (15). Quantitative data on the phospholipids of fungi are rather sparse. In Table IV the quantitative relationship of the phosphatides from *H. grisea* var. *thermoidea* and several fungi are presented. Examination of these data show that the quantitative composition of the lipids of this thermophile differs from the compositions reported for some representative fungi (15-18).

In Basidiomycetes, Deuteromycetes and Phycomycetes, PC and PE are the most abundant phosphatides. In *H. grisea* var. *thermoidea* the quantitatively most important phosphatides were PC, PA and PE in decreasing order of prevalence. The concentration of PA in *H. grisea* var. *thermoidea* far exceeds its concentrations in the other fungi. In *Pythium ultimum* it increases substantially when the temperature is elevated from 20 to 30 C. This high temperature relationship coupled with the high concentrations of PA in the lipids of *H. grisea* var. *thermoidea* suggest that it may be associated with the cultural conditions at elevated temperatures. Whether the high concentration of PA contributes to membrane stabilization or is the result of a temperature dependent change in its metabolism is unclear and needs further elucidation.

The sterols and derivatives comprise an unusually large fraction of the total lipids (16.3-22.9%) and may also be a factor in stability at high temperatures.

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Genetic Variability of Human Plasma and Erythrocyte Lipids[1]

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ABSTRACT

Genetic variation of fasting plasma lipids, lipoproteins and erythrocyte membrane lipids was studied in 67 sets of like-sexed twins and 3 sets of triplets. All of the plasma lipids were more variable in dizygotic twins than monozygotic twins with the exception of phosphatidyl ethanolamine, but only cholesteryl esters, lecithin, phosphatidyl inositol and β-lipoprotein showed significant genetic variation. In contrast, no significant genetic variability was found in any of the erythrocyte membrane lipids and erythrocyte phosphatidyl ethanolamine had significantly greater variation in monozygotic twins. Two sets of twins had an extra lipoprotein band (slow α1); in one family the variant appeared to be segregating as a dominant trait.

INTRODUCTION

Current genetic theory holds that inherited information is passed from generation to generation by deoxyribonucleic acid (DNA) in the nucleus or cytoplasm of the fertilized zygote. The DNA acts as a code which is transcribed into ribonucleic acid (RNA) and translated into proteins, the primary gene products. Lipids are not primary gene products but are genetically controlled through the following mechanisms:

Enzymes

All lipids are synthesized and degraded by a series of reactions catalyzed by enzymatic proteins. Genetic abnormalities of these pathways can result in an accumulation (lipidoses) or deficiency of metabolic products.

Carrier Proteins

Lipids, being relatively insoluble in aqueous solutions are often made more polar and therefore more soluble by complexing them with proteins. Lipid transport by the plasma lipoproteins and the acyl carrier proteins are examples of this mechanism.

Structural Proteins

Biological membranes and connective tissue are formed from protein repeating units but also contain lipids that enable them to perform their functions. In contrast to enzymatic and carrier proteins little is known about genetic variability of structural proteins. The study of lipid variability, especially in cell membranes should provide insight into genetic changes of these proteins.

It is often very difficult to separate the genetic variability due to enzymes, carrier proteins or structural proteins; for example, the enzyme lecithin-cholesterol acyltransferase is a part of plasma α-lipoprotein and interacts with the erythrocyte membrane lipids.

Previous work in this laboratory revealed highly significant between individual variation in plasma and erythrocyte lipids (1). The present study was designed to determine which plasma and erythrocyte lipids have significant genetic variation.

MATERIALS AND METHODS

A total of 67 like-sexed twins and 3 sets of like-sexed triplets were studied ranging in age from 8 to 20 years. All multiple births were like-sexed sets to eliminate variation due to sex. Twin zygosity was determined by blood factors (2) and the probability method described by

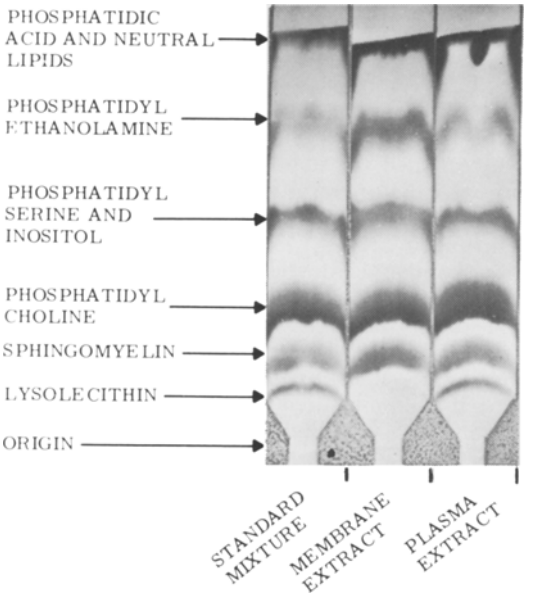

FIG. 1. Thin layer chromatographic separation of phospholipids.

[1]Presented in part at the ISF-AOCS World Congress, Chicago, September 1970.

TABLE I

Analysis of Variance Table for Duplicate Determinations of a Single Sample From Monozygotic and Dizygotic Multiple Births[a]

Source of variance	Degrees of freedom
Among MZ sets	n_1-1
Among DZ sets	n_2-1
Within MZ sets	$\sum_{1}^{n_1} (a_i-1)$
Within DZ sets	$\sum_{1}^{n_2} (b_i-1)$
Duplicate laboratory analyses for individuals	$\sum a_i + \sum b_i$

[a] n_1 is the number of monozygotic twins and triplet sets; n_2, number of dizygotic twins and triplet sets; a_i, number of individuals in each monozygotic set; b_j, number of individuals in each dizygotic set.

Gaines and Elston (3) using blood types A_1, A_2, B, O; M, N, S; C, D, E, c of the Rh series; Fy^a, Fy^b; K, k; P, p; JK^a, JK^b; haptaglobin and phosphoglyceromutase markers by starch gel electrophoresis (4). Using these genetic markers in like-sexed twins the probability of misclassifying a set of dizygotic (DZ) twins into the monozygotic (MZ) groups is less than 1%. Of the 67 twin pairs, 31 were dizygotic and 36 were classified as monozygotic. Two sets of triplets were dizygotic and the remaining set appeared to be monozygotic for a total of 70 sets of multiple births (37 monozygotic and 33 dizygotic).

A single sample of venous blood was collected from each individual in tubes containing disodium ethylenediaminetetra acetic acid (EDTA) 1 mg/ml, following a 12-14 hr overnight fast. Members of each twin or triplet set were sampled and duplicate analyses done concurrently, before results of the the blood typing were known. The blood was immediately centrifuged at 4 C, 2300 g for 30 min. The plasma and erythrocytes were separated and the buffy coat was discarded along with upper 1-2 mm of erythrocytes. The erythrocytes were washed three times in isotonic buffer at pH 7.4 and lipid values expressed per milliliter of packed cells measured by microhematocrit determination. All samples were kept at 1-3 C and analyses done within four days.

All glassware was soaked 12 hr in nitric acid-water (1:1 v/v) and rinsed five times with distilled water. All solvents were reagent grade but not redistilled.

The lipids were extracted in chloroform-methanol with an antioxidant, 2,6-di-tert-butyl-4-methylphenol (Aldrich Chem. Co., Milwaukee) added to the chloroform (5). The lipid phase of the extract was concentrated under nitrogen and brought to a known volume in chloroform for analyses.

Phospholipid separation was done by one-dimensional thin layer chromatogram (TLC) on 0.25 mm layers of Silica Gel H (Brinkman Instruments Inc., Westbury, N.Y.) mixed with 1 mmole Na_2CO_3 solution (6). Before use the plates were marked into six 3 cm lanes with wedge tips (Fig. 1) and activated at 110 C for 1 hr. Lipid extract containing 0.5-1 μmole of phospholipid was spotted one each lane and extract containing approximately 0.2 μmole of phospholipid taken for total lipid phosphorus determination. The chromatograms were developed with chloroform-methanol-acetic acid-water (75:45:12:6 v/v). The developed plates were air dried at room temperature for 20 min and the spots detected by placing the dry plate in an iodine vapor jar for 3 to 5 min (7). After removal from the jar the lipid spots were outlined with a fine needle. Individual phospholipids were identified by the use of standard phospholipids (Supelco, Inc., Bellefonte, Pa.) and the molybdenum blue spray of Dittmer and Lester (8). Using various solvent systems and different concentrations of extract, five phospholipid spots for plasma and four spots for erythrocytes were detected (Fig. 1). Phosphorus determination was carried out on scrapings which were digested with sulfuric acid and hydrogen peroxide (9).

Free cholesterol and cholesteryl esters were separated by TLC on Silica Gel G (Brinkmann Instruments, Inc.) and a solvent made of equal parts chloroform and cyclohexane. The cholesterol and cholesteryl ester spots were identified by iodine vapor and scraped by the same method as used for the phospholipids, extracted in chloroform-methanol (2:1 v/v) and evaporated to dryness. The dried extracts of plasma and erythrocytes were measured for cholesterol content by the method of Abell et al. (10).

Quantitative plasma lipoprotein electrophoresis was done by using the method and equipment of Gelman Instrument Co. (Ann Arbor, Mich.). Plasma samples (40 μl) were electrophoresed on Sepraphore III in a pH 8.8 (tris-barbitol-sodium buffer with 1% bovine albumin and 0.001 M EDTA) for 1 1/2 hr at 20 v and stained with Schiff's reagent. For quantitation the strips were scanned while still moist on a densitometer (Beckman Analytrol with a microzonal adaptor and a 520 mμ filter).

Within twin set mean squares were obtained by the analysis of variance table shown in Table I (11). This analysis allowed unequal subsample

TABLE II

The Means and Standard Deviations of Plasma and Erythrocyte Lipids and Lipoproteins in 37 Sets of Monozygotic and 33 Sets of Dizygotic Twins and Triplets

	Monozygotic	Dizygotic
Plasma		
Total cholesterol (μmole/ml plasma)	3.96 ± 0.52	3.90 ± 0.56
Cholesterol fractions (per cent of total)		
Free cholesterol	29.94 ± 7.83	28.82 ± 6.93
Cholesteryl ester	70.01 ± 13.21	71.19 ± 17.52
Total phospholipids (μmole/ml plasma)	2.33 ± 0.33	2.61 ± 0.34
Individual phospholipids (per cent of total)		
Lysolecithin	5.11 ± 1.78	5.08 ± 1.82
Sphingomyelin	19.32 ± 2.81	20.17 ± 2.91
Lecithin	66.34 ± 3.92	65.71 ± 3.98
Phosphatidyl inositol	2.87 ± 1.04	3.67 ± 1.09
Phosphatidyl ethanolamine	6.20 ± 1.42	6.12 ± 1.69
Lipoproteins (per cent of total)		
Alpha	28.32 ± 10.04	30.13 ± 5.58
Pre-Beta	20.23 ± 14.68	17.40 ± 9.78
Beta	51.44 ± 9.45	52.46 ± 7.43
Erythrocyte		
Cholesterol (μmole/ml red cells)	4.25 ± 0.77	3.84 ± 0.46
Total phospholipids (μmole/ml red cells)	3.97 ± 0.67	3.84 ± 0.49
Individual phospholipids (per cent of total)		
Sphingomyelin	26.79 ± 3.91	25.97 ± 3.84
Lecithin	32.09 ± 5.84	32.24 ± 5.92
Phosphatidyl serine	14.37 ± 2.16	15.05 ± 2.40
Phosphatidyl ethanolamine	26.02 ± 3.92	27.12 ± 3.71

sizes necessitated by inclusion of the triplet data. In this study only the mean squares for within twin sets and laboratory error were used. The among twin sets mean squares were discarded because they contained an unknown amount of variance due to changes in laboratory technique over time. If the within DZ sets mean square was significantly greater than the within MZ sets mean square this was taken as evidence for genetic variation. Because the within-MZ-sets mean square is based on two genetically identical members of a twin pair, it can be assumed to be an estimate of environmental variation. [An exception to identical inheritance of monozygotic twins would be cytoplasmic inheritance which may segregate when a single embryo splits into two embryos.] The within-DZ-sets mean square contains an environmental component but in addition contains genetic variation. The mean squares calculated between duplicate laboratory analyses within individuals are an estimate of laboratory error which is also present in the within twin sets mean squares.

RESULTS AND DISCUSSION

Means and standard deviations for plasma and erythrocyte lipids and plasma lipoproteins of monozygotic and dizygotic twins are shown in Table II. The individual phospholipids, lipoprotein fractions, and free and esterified cholesterol are expressed as a percentage of total phospholipids, plasma lipoproteins and cholesterol respectively; however, statistical analysis was done on the absolute values. The mean values obtained are comparable to previously reported results (1,12-17), and were not significantly different between MZ and DZ sets for any component measured.

Table III shows the within MZ and DZ twin set mean squares and the laboratory error mean

TABLE III

Within Twin Set and Laboratory Error Degrees of Freedom and Mean Squares of Plasma and Erythrocyte Lipids and Plasma Lipoproteins

Lipid Fraction	Mean Squares					
	Within Mz twin sets	DF	Within DZ twin sets	DF	Duplicate laboratory analyses for individuals	DF
Plasma						
Free cholesterol	0.05475	38	0.05876	35	0.00397	143
Cholesteryl esters	0.14778	38	0.53657	35	0.01759	143
Lysolecithin	0.00371	38	0.00452	35	0.00152	143
Sphingomyelin	0.00910	38	0.01331	35	0.00343	143
Lecithin	0.03889	38	0.13953	35	0.00557	143
Phosphatidyl inositol	0.00135	38	0.00297	35	0.00040	143
Phosphatidyl ethanolamine	0.00158	38	0.00123	35	0.00040	143
Lipoproteins						
α-lipoprotein	50.6	20	81.0	20	39.55	80
Pre-β-lipoprotein	29.2	20	55.7	20	18.76	80
β-protein	82.5	20	186.6	20	66.29	80
Erythrocytes						
Cholesterol	0.18156	38	0.20621	35	0.05181	143
Sphingomyelin	0.02312	38	0.03328	35	0.00499	143
Lecithin	0.04001	38	0.02910	35	0.00362	143
Phosphatidyl serine	0.01342	38	0.00898	35	0.00169	143
Phosphatidyl ethanolamine	0.07042	38	0.02251	35	0.00639	143

squares for the 15 lipid and lipoprotein components measured. Laboratory error makes up a large part of the total variation of the minor plasma phospholipids (lysolecithin, phosphatidyl inositol and phosphatidyl ethanolamine) as has previously been reported for these methods (12). Plasma lipoproteins quantitated by these methods also have considerable laboratory error. However, the within MZ and DZ twin sets mean squares were significantly greater than the laboratory error mean squares ($P < 0.01$) for all components except the lipoprotein fractions within MZ twins.

All of the plasma lipids were more variable within DZ twin sets than within MZ twin sets with the exception of phosphatidyl ethanolamine, but a significant DZ/MZ F-ratio was found only in cholesteryl esters ($P < 0.01$), lecithin ($P < 0.01$) and phosphatidyl inositol ($P < 0.05$). In contrast, none of the erythrocyte lipids were significantly more variable in DZ twins when compared to MZ twins. Phosphatidyl ethanolamine which had the lowest DZ/MZ F-ratio of the plasma lipids (0.78) also had an extremely low F-ratio in the erythrocyte lipids (0.32). In fact, erythrocyte phosphatidyl ethanolamine was significantly more variable in MZ than in DZ twins ($P < 0.01$).

In our analysis, we assumed that dizygotic twins were inherently more variable than monozygotic twins since they differ with respect to both genetic and environmental factors. For this reason, a one-tailed significance test was used. However, traits which are determined or influenced by heritable factors in the cytoplasm (18) (such as mitochondria) could be more variable in monozygotic twins.

Lipoprotein analysis was done on only 40 of the 70 sets of multiple births (20 monozygotic and 20 dizygotic twin sets). The only fraction with a significant within DZ/MZ F-ratio was β-lipoprotein ($P < 0.05$), although pre-β-lipoprotein approached significance ($0.1 > P > 0.05$). This could be explained by the fact that pre-β-lipoprotein is partially made up of β-apolipoprotein, therefore, factors which cause genetic variation in β-lipoprotein could cause variation in pre-β-lipoprotein.

The quantitative electrophoresis was done after staining of aldehydes formed from oxidation of double bonds and therefore cannot be expected to be directly proportional to the amount of lipid present. For example, lipids high in unsaturated fatty acids may take up

more stain than saturated lipids.

The MZ and DZ twin sets were not matched for age, sex or time evaluated during the experiment. To determine if these factors gave false indications of genetic variation, the intrapair differences in the lipid values were regressed upon age and sex of the twin sets, but no significant effects were found. Laboratory error mean squares were calculated separately for MZ and DZ individuals and three lipid components (erythrocyte phosphatidyl serine, erythrocyte sphingomyelin and plasma α-lipoprotein) had significantly greater ($P < 0.05$) laboratory error mean squares within MZ twins when compared to DZ twins. None of these lipid components had significant genetic variation.

In addition to the quantitative variation of plasma lipoproteins, 5 of the 80 individuals tested had no detectable pre-β-lipoprotein band and two sets of twins were found to have an extra lipoprotein band migrating between the pre-β and α fraction in the area of previously described slow α-lipoprotein (19) (Fig. 2). One of these sets, monozygotic girls had no other family member with the slow α band, but the second, dizygotic girls had a sister and their mother with the extra α band.

At least five previous twin studies of plasma lipids have been reported (20-24). Osborne et al. (20) studied 82 twin pairs and found that monozygotic female twins living apart had significantly greater variation in plasma cholesterol than similar twins living together. He also compared monozygotic and dizygotic twins but found no significant intrapair differences for cholesterol, cholesteryl esters and total lipid phosphorus.

Chin (21) studied only 15 twin pairs and had a larger intrapair variation for plasma sphingomyelin within dizygotic twin pairs as compared to monozygotic twin pairs, but only when sphingomyelin was expressed as a percentage of total lipid phosphorus. This same relationship was not present when the sphingomyelin was expressed in absolute terms. Chin's paper is difficult to interpret because he arbitrarily classed each set of twins "A" and "B" and used the differences between A and B twins as an estimate of intrapair variance when in reality this variance estimate is only a random fraction of the intrapair variance.

Gedda and Poggi (22) in 1960 and McDonough et al. (23) in 1962 both found significantly greater dizygotic variation in plasma cholesterol than monozygotic twins. Jensen et al. (24) in 1965 found significantly greater dizygotic intrapair variance for cholesterol, phospholipid and triglycerides when compared to monozygotic twins. These same

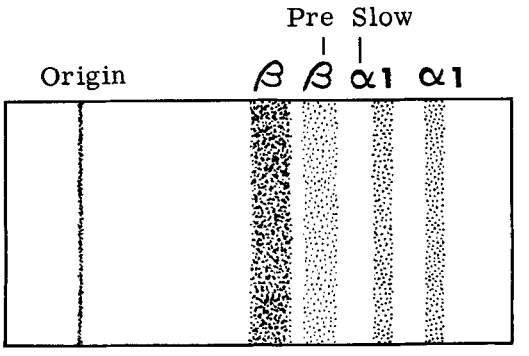

FIG. 2. Drawing of cellulose acetate lipoprotein electrophoresis showing slow moving α_1 band.

workers also found heterogeneity among monozygotic twin pairs which they felt was too large to be attributed to environment. Using Fisher's test for heterogeneity among twin pairs (25) they were able statistically to divide monozygotic twins into two groups, one with close intrapair agreement of lipid values and another with marked discrepancies. They were unable to postulate any biological basis for this heterogeneity; however, Nance (18) has postulated that discordance of monozygotic twins for apparently inherited traits may be explained by segregation of cytoplasmic DNA at the time the single embryo splits into twins.

The present study confirms the presence of significant genetic variation in plasma as reported by the previous workers (20,21,24). Unlike Jensen et al. (24) the present study found no significant genetic variation in free cholesterol but did find highly significant genetic variation in the plasma cholesteryl ester fraction.

Previous reports of significant genetic variation in plasma phospholipids were also confirmed and in the present study this variation was explained by significant genetic variation in lecithin and phosphatidyl inositol.

The plasma lipoprotein study revealed that β-lipoprotein had significant genetic variability but not α- and pre-β-lipoproteins. To investigate the possibility that all of the plasma lipid genetic variations was a reflection of variation in β-lipoprotein, a covariance analysis was done and lecithin, phosphatidyl inositol and cholesteryl esters were still found to have significant genetic variation. The present study confirmed the heritable nature of plasma lipids but genetic variability could not be detected in the erythrocyte membrane. Erythrocyte membrane lipids are known to be in a state of dynamic equilibrium with the plasma lipids (26) and this

equilibrium is influenced by environmental factors (27). This brings up the possibility of using erythrocyte lipid content as a "genetically unbiased measure" of plasma lipid metabolism.

There are many questions to be answered, for example, what part of the genetic variation in β-lipoprotein is due to variation in the amount or lipid binding capacity of its apoprotein, variation in the anabolism or catabolism of the lipid components or interaction of these factors? More importantly, methods must be devised to relate genetic variation in plasma lipids and lipoproteins to desease states, most notably atherosclerosis, so that persons with a genetic tendency twoard atherosclerosis may be identified early in life.

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Metabolic Studies in Isolated Rat Liver Cells: I. Lipid Synthesis

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ABSTRACT

Rat liver cells, isolated by chelate perfusion and extrusion through a tissue press, incorporated labeled acetate into cellular lipids and into lipids released into the suspending medium. Optimal rates of incorporation required supplementation of tris-KCl medium with Mg^{++}, Mn^{++}, succinate, citrate, nicotinamide, Coenzyme A, NADP and glucose-6-phosphate. The rate of acetate incorporation was markedly altered by changes in incubation media; tris-KCl was the most effective buffer. All the major classes of cellular lipids were labeled. ATP, BSA, inorganic phosphate, Ca^{++}, 2,4-dinitrophenol and sodium clofibrate were potent inhibitors of acetate incorporation. When added to the incubation mixture, several hormones altered the rate of acetate incorporation into lipids.

Studies with subcellular fractions from liver have shown that fatty acid synthesis takes place both in mitochondria (1) and in the particle-free fraction (2,3), whereas complex lipid formation occurs in microsomes (4). The use of isolated subcellular fractions, however, precludes evaluation of the interplay between cellular organelles. Several studies have shown that such interactions play an important role in the control of lipid synthesis. The addition of mitochondria to the soluble fraction of pigeon liver markedly stimulated fatty acid synthesis, replaced several cofactor requirements, and greatly altered the composition of the synthesized fatty acids (5). The stimulation of fatty acid synthesis by the addition of microsomes and glycerol-3-phosphate to the supernatant fraction of rat liver suggests that the rate of fatty acid formation may be regulated in part by the removal of fatty acyl CoA to form complex lipids (6). Moreover, broken cell preparations have probably lost the level of organization necessary to respond to physiological stimuli such as hormones. Hence, delineation of the factors which regulate hepatic lipid synthesis requires a simple tissue preparation that also retains enzyme localization and a high degree of cytoarchitectural organization.

Isolated liver cells may provide such a preparation. The present study was undertaken to characterize the lipid biosynthetic capacity of dispersed liver cells, obtained in excellent yield by a rapid, simple method. The results of these studies indicate that liver cells, suspended in a suitably supplemented incubation medium, incorporated ^{14}C-acetate into fatty acids and complex lipids, and that the rate of lipogenesis was altered by the addition of various hormones to the incubation medium. Demonstration of cellular lipogenesis was necessary to indicate the feasibility of using this preparation for subsequent study of serum lipoprotein synthesis.

MATERIALS AND METHODS

Materials

Glucagon, Coenzyme A (lithium salt), and the sodium salts of NADP, ATP and glucose-6-phosphate were purchased in A grade from Calbiochem. Puromycin dihydrochloride (grade II), cycloheximide, Trizma (tris), reduced glutathione, 2,4-dinitrophenol, 17-beta-estradiol, L-thyroxine and ACTH were obtained from the Sigma Chemical Company. Cholesterol and nicotinamide were supplied by Eastman Organic Chemicals. Digitonin and sodium succinate were obtained from the Fisher Scientific Company; bovine serum albumin (fraction V) from the Mann Research Laboratories, Inc.; and PPO from the Packard Instrument Company. POPOP, 1-^{14}C-sodium acetate (2.0 mc/mM), and 4-^{14}C-cholesterol (57 mc/mM), were bought from the New England Nuclear Corp. Sodium alpha-parachlorophenoxyisobutyrate (sodium clofibrate) was an investigational product provided by Ayerst Laboratories. Crystalline zinc insulin was obtained from E.R. Squibb and Sons; dexamethasone phosphate from Merck, Sharp and Dohme; and L-epinephrine from Parke, Davis. All other reagents were products of the J.T. Baker Chemical Company.

Preparation of Liver Cells

A slight modification of the method of Ontko (7) was used to prepare liver cells from 275 g female Sprague-Dawley rats that were fed Purine Laboratory Chow and lib. Rats were anesthetized with ether, and 50 ml of warm (37

TABLE I

Cofactor Requirements for Incorporation of ^{14}C-acetate Into Cellular Lipids[a]

Cofactor components	Relative incorporation of ^{14}C-acetate, %
Complete cofactor mixture[b]	100
$MgCl_2$	9
$MnCl_2$	70
Sodium succinate	83
Coenzyme A	25
Sodium citrate	39
Nicotinamide	34
NADPH generating system[c]	65
All cofactors	0.3

[a]Cells were incubated under air at 37 C for 2 hr in stoppered 25 ml Erlenmeyer flasks in a water-bath shaker at 130 oscillations per minute. Each flask contained 4 to 6x10^6 cells (0.65 ml concentrated cell suspension); 1-^{14}C-acetate (1.5μc); potassium penicillin G (1 mg/ml); streptomycin sulfate (0.03 mg/ml); tris-HCl (20 mM), pH 7.3 (37 C); KCl (100 mM); and other components as indicated in a total volume of 3 ml. The various cofactors were individually prepared and separately added to the flasks in a total volume of 0.10 to 0.15 ml. The complete cofactor mixture was added as 0.1 ml of a concentrated solution of all the above cofactors. With each experiment one flask, which contained all components except the label, was incubated for the same period and placed on ice. Cold sodium acetate and labeled acetate were added; the cellular lipids were extracted and the radioactivity of this sample was subtracted from that of the other samples to determine acetate incorporation into lipid.

[b]The final concentration of the components of the complete cofactor mixture are: $MgCl_2$ (3.3 mM), $MnCl_2$ (0.1 mM), sodium succinate (10 mM), Coenzyme A (0.03 mM), sodium citrate (3.3 mM), nicotinamide (6.7 mM), NADP (0.23 mM) and glucose-6-phosphate (1.7 mM).

[c]NADP and glucose-6-phosphate.

C) calcium-free Locke's citrate solution was forcibly infused into the clamped portal vein with a 50 ml syringe and 22 gauge needle inserted about 1 cm from the liver. After the initial perfusion of about 30 ml of warm solution over a 25-35 sec period, the liver appeared blanched and distended. The hepatic veins were then severed to decompress the liver and to prevent circulatory backflow, and the remaining 20 ml was rapidly injected. The rinsed liver was extruded through a tissue press and dispersed in 50 ml of cold (4 C) Locke's citrate solution. This mixture was filtered through 100 mesh silk cloth, and the filtrate was centrifuged at 100 x g for 5 min, resuspended, and recentrifuged as described (7). The packed cells were gently suspended with an equal volume of cold tris (0.02 M) - KCl (0.1 M), pH 7.8 at 4 C (subsequently termed tris-KCl solution) to form the concentrated cell suspension used for study. In every experiment cells were counted with a hemocytometer.

Prevention of Bacterial Contamination

Before use, all solutions were passed through a 0.45μ Millipore filter into glass vessels preheated at 180 C for 15 min, and penicillin (1 mg/ml) and streptomycin (0.03 mg/ml) were routinely included in the incubation mixtures. Periodic bacterial counts on blood agar plates revealed a maximum of 10 organisms per ml at the end of 2 hr of incubation.

Lipid Extraction and Determination of Radioactivity

At the end of each incubation period the flasks were placed on ice, and 0.2 ml of a 10% solution of unlabeled sodium acetate was added to every flask. Cells and medium were separated by centrifugation at 2000 x g for 10 min at 4 C. The medium was either discarded or lyophilized to dryness for subsequent lipid extraction. Packed cells and dried medium residues were extracted by the method of Folch et al. (8). Extraction of lipids from cells by this method was at least 95% complete as determined by reextraction of cellular residues. A measured aliquot of washed lower phase containing labeled lipids was transferred to a glass counting vial, gently dried on a hot plate, dissolved in 10 ml of scintillation fluid (0.5% PPO, 0.01% POPOP in toluene), and counted in an Ansitron liquid scintillation spectrometer. The efficiency of the scintillation spectrometer was 58%.

RESULTS

Cell Yield and Morphological Characteristics

Under optimal conditions, 9.0-12.5 x 10^6 cells were recovered per gram of liver. The presence of the chelating agent during perfusion was essential since replacement of Locke's citrate with saline resulted in drastic reduction in cell yield and the persistence of large cell clumps. When examined either unstained or after staining with hematoxylin and eosin, the cells appeared morphologically intact and extracellular debris was minimal. More than 90% of the preparation consisted of single cells with occasional clumps of up to four cells. On electron microscopic examination of cells fixed with 3% glutaraldehyde solution followed by 2% osmic acid solution, there were small interruptions in the plasma membranes, dilatation of the cisternae of the endoplasmic reticulum, and increased density of the mitochondrial matrices. Ribosomes remained attached to the membranes of the rough endoplasmic reticulum, and intracellular membranes were intact.

Observations During Incubation of Cells

In a few experiments cells were counted initially and at 20 min intervals for 3 hr during

TABLE II

Effect of Incubation Media on ^{14}C-Acetate Incorporation Into Cellular Lipids[a]

Medium	Relative incorporation of ^{14}C-acetate, %
Tris-KCl	100
Tris-NaCl	62
Tris-sucrose	67
Tris (0.001 M)-Sucrose (0.06 M)	38
Hanks' solution (calcium-free)[b]	42
Locke's citrate (calcium-free)	8
Sodium phosphate (0.1 M)	2
Potassium phosphate (0.1 M)	1
Ringer's lactate[c]	0

[a]Incubation conditions are as described for the complete cofactor mixture in Table I, except for substitution of the indicated buffers for tris-KCl. the top three media were 0.02 M in tris and 0.1 M in the other component. The pH at the beginning of incubation was 7.3 for all media.

[b]Contains, per liter, 8 g NaCl, 0.4 g KCl, 0.2 g $MgSO_4 \cdot 7\ H_2O$, 0.06 g $Na_2HOP_4 \cdot 2\ H_2O$, 0.06 g KH_2PO_4, and 1 g glucose.

[c]Contains, per liter, 6 g NaCl, 0.3 g KCl, 0.2 g $CaCl_2$, and 3.1 g sodium lactate.

incubation. There was no significant reduction in the number of intact cells with time. However, the cells displayed a tendency to aggregate to a variable degree after 30 min of incubation. Aggregation was minimal when the oscillation rate was maintained at 130/min and could be completely prevented, even at lower oscillation rates, by the addition of 1 mM $CaCl_2$. Calcium was not used in these studies since it completely prevented acetate incorporation when added in concentrations of 1 mM or higher. Determination of the protein content of the cells and medium (9) at the beginning and end of incubation revealed an initial leakage of 20% of cellular protein upon dilution of the concentrated cell suspension in incubation medium. However, no further leakage of protein occurred during the incubation period.

Cofactor Requirements

No significant incorporation of labeled acetate occurred in hepatocytes suspended in tris-KCl solution. Therefore, a number of cofactors found essential for lipid synthesis by subcellular fractions of liver (2,3) were tested in similar concentrations for their effect on acetate incorporation by liver cells. The results in Table I demonstrate that a combination of several of these cofactors was necessary for active incorporation of acetate by hepatocytes. Omission of Mg^{++} or Coenzyme A was particularly detrimental to acetate incorporation, but all constituents were required for maximai activity. When added individually to cells suspended in tris-KCl medium, none of the cofactor components of the complete mixture significantly enhanced the rate of ^{14}C-acetate incorporation. Glutathione (3 mM) had no effect on cellular acetate incorporation although an absolute requirement for a sulfhydryl compound in fatty acid synthesis by the pigeon liver cytosol has been described (3).

Effect of Removal of Kupffer Cells

In two experiments, the reticuloendothelial cells were first removed magnetically after intravenous injection of powdered iron (7). The resultant parenchymal cell preparation incorporated acetate as actively as rat liver cells prepared without such pretreatment. This data eliminated the necessity of routine prior removal of the Kupffer cells, as did the finding (10) that methods that use a combination of a chelating agent and mechanical disaggregation yield predominantly parenchymal cells.

Effect of Cell Washes

Cell preparations were routinely subjected to a resuspension step in order to reduce tissue debris and minimize the possibility of acetate incorporation by extracellular enzymes or free subcellular particles. This wash procedure had little effect on the cellular capacity for lipid synthesis. Nor did the addition of the initial tris-KCl supernatants to the washed cells stimulate acetate incorporation. Hence, little or none of the measured incorporation resulted from the activity of soluble enzymes or subcellular particles released from damaged cells. Similarly, in our other studies, addition of dispersion or wash fluid to washed cells had no significant effect on rates of amino acid incorporation into cellular protein.

TABLE III

Effect of Inhibitors on ^{14}C-Acetate Incorporation Into Cellular Lipids[a]

Inhibitor	Inhibition of ^{14}C-acetate incorporation, %
$KHCO_3$(2.5 mM)	58
ATP (0.8 mM)	15
(3 mM)	87
(4 mM)	97
Puromycin (2 mM)	21
Cycloheximide (1.7 mM)	45
Sodium clofibrate (3 mM)	96
Phosphate (10 mM)	59
(12 mM)	66
2,4-Dinitrophenol (0.25 mM)	100
Fat-poor BSA (4%)	99
Calcium (1 mM)	100

[a]Incubation conditions are as described for the complete cofactor mixture in Table I.

Incubation Media

Acetate incorporation into lipids was compared in various media supplemented with the lipid cofactor mixture (Table II). Tris-KCl was clearly superior to the other buffers tested. The inability of equimolar tris-NaCl or tris-sucrose solutions to match tris-KCl in support of cellular lipid synthesis illustrates the importance of K^+ to this process. As further shown in Table II, reduction in the ionic strength of the tris-sucrose solution was accompanied by a decrease in acetate incorporation by liver cells. Acetate incorporation in Hanks' solution was comparable to that in hypotonic tris-sucrose medium, while cellular acetate incorporation was minimal in Locke's citrate solution, 0.1 M phosphate buffer, or Ringer's lactate. These findings demonstrate the marked dependence of cellular acetate incorporation upon the composition of the incubation medium.

Inhibitors of Acetate Incorporation

Certain substances tested as potential cofactors for lipid synthesis proved instead to be inhibitory (Table III). The inhibitory effect of ATP was not reversed by the addition of Mg^{++}, and therefore was not due simply to Mg^{++} chelation. The inhibition by bicarbonate and ATP is consistent with the observation that low concentrations of cofactors enhanced acetate incorporation in subcellular liver fractions while higher concentrations had the opposite effect (11). Bicarbonate was present in the preparative media while ATP is endogenously generated by mechanically prepared liver cells (12). Puromycin and cycloheximide were moderately inhibitory, while phosphate, Ca^{++}, BSA, 2,4-dinitrophenol and clofibrate markedly inhibited acetate incorporation.

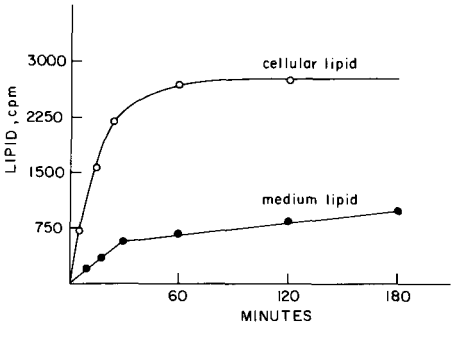

FIG. 1. Time course of 1-^{14}C-acetate incorporation into lipids of the cells and of the extracellular medium. Incubation conditions are as described for the complete cofactor mixture in Table I.

Time Course of Acetate Incorporation

As shown in Figure 1, isolated liver cells incorporated ^{14}C-acetate into cellular lipids at a rapid rate during the first 30 min of incubation. The rate of incorporation then fell progressively and reached a plateau by 2 hr. Labeled lipids appeared in the medium during the first 5 min of incubation and were released at a rapid linear rate for the next 25 min. After 30 min the rate of release of labeled lipids slowed but remained linear for an additional 150 min. After 1 hr of incubation, the labeled lipids in the extracellular medium constituted 20% to 25% of the total labeled lipids in the incubation mixture, and probably represented the lipid portion of serum lipoproteins released by the liver cells (13).

Distribution of Label in Subcellular Fractions

The results of subcellular fractionation (14) of the liver cells demonstrate that more than 80% of the labeled lipids were recovered in the nuclear and mitochondrial fractions (Table IV). The microsomal fraction contained only abou

TABLE IV

Distribution of Radioactivity in Subcellular Fractions[a]

Cell fraction	Total radioactivity	Relative distribution, %
Nuclei, cell debris	4566	49.5
Mitochondria	3177	34.4
Microsomes	893	9.7
Supernatant	581	6.3

[a]Incubation conditions are as described for the complete cofactor mixture in Table I. After 2 hr of incubation, the cells from an entire liver were homogenized in 6 ml of fresh tris-KCl buffer, 2 ml of 1 M sucrose solution was added, and subcellular fractions were separated by the method of Hogeboom et al. (14).

10% of the labeled lipids. However, subcellular fractions obtained from lower speed centrifugations contain significant quantities of attached microsomes (15).

Distribution of Label Among Lipid Classes

It was important to define the distribution of incorporated acetate among the major lipid classes. A combination of hexane-diethyl ether-glacial acetic acid (70:20:1 by volume) was used to separate the labeled lipids of cells and medium by thin layer chromatography (TLC). The results in Table V present the typical, reproducible distribution of label among the lipid classes of liver cells and medium using this solvent system. The percentage of label in phospholipids, monoglycerides, cholesterol and fatty acids was very similar for both cells and medium, while the proportion of label in 1,2-diglycerides was much higher in the medium than in the cells. The quantity of labeled triglycerides and cholesterol esters in the medium was extremely small.

Since the 1,3-diglyceride and cholesterol standards cochromatographed by this method, the incorporation of label into cellular cholesterol was corroborated by digitonin precipitation, using a modification of the method of Crawford (16). The data in Table VI show that cholesterol radioactivity determined by TLC correlated well with that obtained by digitonin precipitation. In another rat used for comparison of these two methods, the percent of total lipids as cholesterol was 8.2% by digitonin precipitation and 5.8% by TLC.

Effect of Hormones

Several hormones, when added individually to liver cell incubations, altered the rate of ^{14}C-acetate incorporation into cellular lipids

TABLE V

Distribution of Radioactivity Among the Various Lipid Classes[a]

	Distribution of radioactivity			
	Cells, 79%		Medium, 21%	
Lipid classes	cpm	%	cpm	%
Phospholipids	3847	35	998	35
Monoglycerides	1875	17	478	16
1,2-Diglycerides	619	6	522	18
Cholesterol	958	9	195	7
Fatty acids	2524	23	626	22
Triglycerides	938	9	29	1
Cholesterol esters	275	2	7	0
Total	11036		2855	

[a]Incubation conditions and concentrations of cells and cofactors were unchanged from those described in Table I except that the quantity of incubation material was five times that usually employed and the concentration of label was doubled. Cells and medium were separated, extracted and washed as described in Methods. The cellular and medium lipids were resolved by TLC using hexane-diethyl ether-glacial acetic acid (70:20:1 by volume) into the above fractions, listed in the order of their distance from the origin. The spots were identified, scraped from the same plate, and counted as before. The radioactivity in the table represents that derived from 3/10 of the entire incubation mixture. Total lipid radioactivity present in the aliquot of extracted cellular lipid applied to the coated plate was 16,416 cpm.

TABLE VI

Comparison of Digitonin Precipitation and TLC for Determination of Labeled Cholesterol in Liver Cells

Method	1-^{14}C-acetate incorporation into cholesterol[a]		
	Radioactivity		Proportion of total labeled lipids as cholesterol, %
	Observed, cpm	Corrected for recovery, cpm	
Digitonin precipitation	986	2380[b]	14.5
TLC	958	1475[c]	9.0

[a]Cholesterol label was determined by both digitonin precipitation using carrier cholesterol and TLC in identical aliquots of the extracted cellular lipids. Total lipid radioactivity present in each aliquot of cellular lipid extract was 16,416 cpm as in Table VI.

[b]The recovery of cholesterol was determined by control samples in which a known amount of labeled cholesterol was added to carrier cholesterol prior to addition of digitonin.

[c]Calculation of recovery of labeled cholesterol was made by division of the total lipid cpm recovered after TLC by the quantity of radioactivity applied to the plates (16,416 cpm).

(Table VII). Dexamethasone and L-thyroxine stimulated acetate incorporation, while epinephrine and 17-beta-estradiol were inhibitory. Insulin was usually mildly inhibitory while ACTH and glucagon were without measurable effect. The changes in labeled lipid in the medium paralleled the incorporated label in cellular lipid for both dexamethasone and epinephrine. This finding indicates that these hormones did not change the quantity of labeled lipid present in cells by altering rates of lipid secretion by the cells.

DISCUSSION

Isolated liver cells have several distinct advantages as a preparation for metabolic studies. Hepatocytes are sufficiently organized to respond to physiological stimuli such as hormones (17); yet they are less complex than liver slices and have a larger exposed surface area. Moreover, the availability of a large number of identical cell aliquots from a single liver and of techniques for separation of reticuloendothelial cells from parenchymal cells (7,18) provide further advantages in metabolic studies.

However, suspended liver cells do present certain inherent drawbacks for the study of biochemical regulation. Their principal disadvantages stem from damage to the plasma membrane incurred during cell separation. The resulting alteration in membrane permeability leads to leakage of various soluble enzymes, cofactors and ions into the preparative and suspending media (19). The loss of soluble glycolytic enzymes impairs endogenous respiration by hepatocytes and prevents the utilization of glucose (19). However, because particulate mitochondrial enzymes are retained, the addition of succinate markedly stimulates cellular oxygen consumption (20) and probably results in enhancement of lipid synthesis by its utilization as a respiratory substrate. Finally, there are ultrastructural changes which vary in degree with the gentleness of the method selected for cell preparation.

Leakage is not confined to isolated cells since the egress of intracellular protein also occurs during incubation of liver slices, both from damaged cells at the surface of the slice and from intact cells within the tissue (21). In contrast, the loss of soluble protein from liver cells occurs during the dispersion step of the preparation (22), and we have found, as did others (23), that no further leakage of protein into the medium occurs during incubation.

The capacity of dispersed liver cells to incorporate ^{14}C-acetate into lipids demonstrates that they retained sufficient soluble enzymes to allow investigation of processes mediated in part by enzymes of the soluble phase. Further, the stimulatory effects of cofactors demonstrates the feasibility of adding back missing substances under controlled conditions for assessment of their relative importance.

Reports of fatty acid synthesis in subcellular fractions of liver (1-6) have outlined various requirements for active lipogenesis. In general, it was observed that as crude soluble extracts of pigeon liver underwent successive purification steps, several new requirements emerged (3), and very little synthesis occurred when any of

TABLE VII

Effects of Hormones on ^{14}C-Acetate Incorporation Into Cellular Lipids[a]

Hormone present	Final concentration, M x 10^5	Relative incorporation of 1-^{14}C-acetate into cellular lipids
None		1.00
Dexamethasone	2.0	1.53 ± 0.22[b] (5)[c]
L-thyroxine	3.9	1.20 (2)
Epinephrine	1.8	0.62 ± 0.08 (3)
17-beta-estradiol	0.25	0.71 ± 0.04 (3)
ACTH	0.063	0.97 (1)
Glucagon	0.34	0.98 (2)
Insulin[d]	8.3 microunits/ml	0.86 ± 0.14 (4)

[a]Final dilutions of hormones were carried out just before incubation of cells. Hormones were individually added to mixtures of cofactor-supplemented medium and liver cells which were kept at 4 C until incubation was begun. The complete cofactor mixture and label were present from the beginning of incubation. Cells were incubated for 2 hr as described under Table I.

[b]Mean ± standard deviation.

[c]Number of animals studied in parentheses.

[d]Sodium pyruvate (0.3 mM) was used together with insulin.

these cofactors was omitted. The formation of fatty acids in suspended rat liver mitochondria also required certain cofactors (1).

Cytoplasmic preparations of liver have consistently required Coenzyme A, NADPH, and Mg^{++} or Mn^{++} for fatty acid synthesis. In the present work the combination of Mg^{++} and Mn^{++} was more stimulatory to acetate incorporation than either ion alone. The combination of NADP and glucose-6-phosphate provided an NADPH-generating system for fatty acid production. Coenzyme A was required for synthesis of fatty acids, complex lipids and cholesterol. The beneficial effect of nicotinamide on lipid synthesis probably resulted from its sparing action on residual intracellular NAD since nicotinamide is known to inhibit splenic NADase at similar concentrations (24), and dispersed liver cells are deficient in nicotinamide nucleotides (19).

The superiority of tris-KCl over equimolar tris NaCl in support of lipogenesis parallels its supportive role in cellular respiration (7), and may result from the enhanced rate of mitochondrial ATP synthesis in the presence of potassium (25). Although unfortified tris-KCl promoted ketogenesis by liver cells (7) the presence of cofactors in the present study may channel acetate into the assembly of fatty acids instead of into ketone bodies. The lipogenic effect of K^+ may be simply due to replacement of intracellular K^+ which has leaked from the cells during their preparation (26).

The adverse effects of Ca^{++} and phosphate on lipid synthesis probably result from their abnormal entry into the cell because of loss of selective permeability of the plasma membrane. Both ions can interfere with O_2 utilization by liver cells (7,19,20) perhaps by an uncoupling of mitochondrial oxidative phosphorylation (27). The failure of cells suspended in Ringer's lactate to incorporate acetate is probably related to the Ca^{++} ion content of this medium. Inhibition by 2,4-dinitrophenol demonstrates the need for continuous generation of high energy phosphate despite the inhibition by externally added ATP.

The inhibitory effect of exogenous ATP on acetate incorporation by liver cells is similar to that reported in subcellular hepatic preparations (2,28). ATP may prevent the activation of acetyl CoA carboxylase by citrate (28) or may reverse the cytoplasmic stimulation of long chain fatty acyl-CoA synthetase in rat liver (29).

The anti-lipogenic effects of sodium clofibrate deserve further careful study since a specific inhibitor of lipid synthesis might provide an important tool for elucidation of the role of lipid synthesis in cellular metabolism. Moreover, the dramatic inhibition of ^{14}C-acetate incorporation by this agent suggests that dispersed liver cells may be useful for testing the effectiveness of pharmacologic agents whose site of action is the liver.

The present finding that radioactive cholesterol accounted for 6% to 9% of the total label in all lipid classes contrasts with the conclusion of Lata and Reinertson (30) that incubated liver cells were unable to synthesize cholesterol despite the addition of CoA, ATP and NADH and with the data of Ichihara et al. (17) who reported that labeled cholesterol constituted only 1.6% of the total radioactivity in cellular lipid.

Large amounts of labeled diglyceride and

minimal quantities of labeled triglyceride were present in the incubation medium, but in the cells similar amounts of label were found in diglyceride and triglyceride. The large proportion of labeled diglyceride in the incubation medium is difficult to explain since hepatic glyceride is normally exported into the plasma as triglyceride. It is possible that a lipase present in the plasma membrane of rat hepatocytes (31) hydrolyzed triglyceride to diglyceride during incubation. The absence of labeled cholesterol esters in the incubation medium is consistent with recent evidence (32) that circulating cholesterol is largely esterified in the plasma itself by an acyltransferase reaction rather than in the tissues.

The changes in cellular rates of lipogenesis in response to hormonal additions provide evidence for the functional integrity of isolated liver cells. The inhibition of acetate incorporation observed with epinephrine is in agreement with previous findings in slices (33). The reduction in incorporation found with 17-beta-estradiol concurs with the reported inhibition of cholesterol synthesis in microsomes from estrogen-treated rats (34). The stimulation of lipid synthesis found with L-thyroxine was consistent with a similar enhancement that occurred in hepatic slices of rats pretreated with L-thyroxine (35). The lipogenic effect of dexamethasone on acetate incorporation is interesting in view of the protective activity of this hormone on the plasma membrane of isolated hepatocytes (22). In the present study, the addition of dexamethasone may have promoted retention of the soluble fatty acid-synthesizing enzymes within the cellular cytoplasm. The harmony of these results on hormonal effects on lipid synthesis with those obtained using other preparations of liver from hormone-injected rats suggests the potential utility of the isolated liver cell preparation for future metabolic studies.

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Metabolic Studies in Isolated Rat Liver Cells: II. Biosynthesis of Serum Low Density Lipoproteins and its Regulation

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ABSTRACT

Dispersed rat liver cells were used for study of protein and serum lipoprotein synthesis. Cellular leucine incorporation was tested in the presence of various cofactors, buffers and inhibitors. ^{14}C-leucine was incorporated into cellular protein at an active rate for 1 hr. Incorporation was more rapid in the presence of succinate, $MgCl_2$, phosphate and nicotinamide, but these cofactors were not absolutely required. The liver cells also incorporated labeled leucine into lower density lipoproteins (lDL) and released the newly labeled lDL into the incubation medium. Puromycin strongly inhibited the production of cellular protein and lDL. The mode of cellular regulation of lDL synthesis was examined. The rate of lDL production was selectively enhanced by short term alimentation with dietary triglyceride or by the addition of a mixture of lipogenic cofactors to incubated liver cells. The utility of isolated liver cells as a preparation for metabolic control studies is discussed.

Studies of serum lipoprotein synthesis have utilized liver preparations that ranged in organizational complexity from subcellular particles (1,2) to the perfused intact organ (3). Product identification has been verified by peptide mapping (4) and by immunochemical techniques (2).

The present work was undertaken to define the characteristics of an hepatic preparation that would be suitable for study of serum lipoprotein formation and the metabolic regulation of this process. Isolated liver cells respire (5) and retain subcellular organization and the biochemical capacity for synthesis of lipids (6), proteins (7,8) and RNA (7). Previous work had shown that the metabolic properties of liver cells varied with the method of cell preparation (9). Therefore, it was necessary to determine the characteristics of general protein synthesis in hepatocytes isolated by our method preliminary to study of the synthesis of serum lower density lipoproteins (lDL). We define the term lDL to include both very low density lipoproteins (VLDL) and low density lipoproteins (LDL).

The present report describes optimal conditions for protein synthesis and indicates that suspended liver cells synthesized lipoproteins with the ultracentrifugal and immunological properties of rat lDL. Moreover, the rate of this process was altered by in vivo and in vitro manipulations. A preliminary report of this work has been presented (10).

MATERIALS AND METHODS

Materials

Ediol (Lipostrate-CB) and GTP (sodium salt) were purchased from Calbiochem; trisodium phospho(enol) pyruvate and pyruvate kinase (type II) from Sigma; and Triton X-100 from Rohm and Hass, Inc. U-^{14}C-L-leucine (273 mc/mM) and 1-^{14}C-sodium acetate (2 mc/mM) were products of the New England Nuclear Corp. Freund's complete adjuvant and Difco special noble agar were obtained from Difco Laboratories; agarose from Fisher Scientific Company; rabbit antiserum to whole rat serum and goat antiserum to rabbit gamma globulin from Hyland Laboratories; and crystalline egg albumin from the Nutritional Biochemical Corp. The sources of all other reagents have been described (D.M. Capuzzi and S. Margolis, submitted to Lipids).

Preparation of Liver Cells

Liver cells were prepared as described previously (D.M. Capuzzi and S. Margolis, submitted to Lipids). Similar precautions against bacterial contamination were taken.

Isolation of Rat Serum lDL

The density of serum or of incubation medium was raised to the desired nonprotein solvent density by addition of a concentrated salt solution according to the formula of Havel et al. (11). Rat serum was adjusted to d 1.063 and centrifuged at 100,000 x g for 16-18 hr in a Model L Spinco preparative ultracentrifuge. The top 1.5-2.0 ml of each tube was removed

TABLE I

Effect of Various Additions on ^{14}C-leucine Incorporation Into Cellular Protein[a]

Additions	Relative incorporation of ^{14}C-leucine, %
None	100
Succinate	139
Magnesium chloride	245
Phosphate	138
Nicotinamide	120
Combined cofactors	338

[a]Cells were incubated under air at 37 C for 2 hr in stoppered 25 ml Erlenmeyer flasks in a water bath shaker at 130 oscillations/min. Each flask contained 4 to 6 x 10^6 cells (0.65 ml of concentrated cell suspension); ^{14}C-L-leucine (1.25 μc); potassium penicillin G (1 mg/ml); streptomycin sulfate (0.03 mg/ml); tris-HCl (20 mM), pH 7.3 at 37 C; KCl (100 mM), and other components as indicated in a total volume of 4 ml. Where indicated, additions were made to give the following final concentrations: $MgCl_2$ (7.5 mM); potassium phosphate (10 mM); nicotinamide (7.5 mM); sodium succinate (12 mM). Combined cofactors consisted of all four additions. In each experiment one flask, which contained all components except the label, was incubated for the same period and placed on ice. Cold leucine and labeled leucine were then added. The amount of radioactive protein in this sample was subtracted from that of the other samples to determine leucine incorporated into protein. Results are averages of five experiments.

by careful aspiration with a Pasteur pipet. For use as carrier lDL, this upper fraction was dialyzed for 3 hr against 0.15 M NaCl solution containing 0.01% disodium EDTA at pH 7.4. For rabbit inoculations, the density of the material was readjusted to d 1.035 and recentrifuged for 16 hr at 100,000 x g. The top 1.5 ml were removed and used for inoculation.

Inoculation of Rabbits

Rabbits received weekly subcutaneous injections of lDL isolated at d 1.035. Each antigen inoculation contained about 2 mg lDL protein emulsified with Freund's adjuvant. The antiserum obtained after four to six injections was stored at -20 C until use. Antisera against chicken ovalbumin were obtained from other injected rabbits.

Separation of Medium From Cells and Determination of Labeled Cellular Protein

Incubation conditions are described under Table I. Five minutes before the end of each incubation period, 0.2 ml of a 2% solution of unlabeled L-leucine and 1 ml of rat carrier lipoprotein (2 mg protein) were added to every flask. The contents of each flask were centrifuged at 100 x g for 5 min at 4 C. The packed cells were gently washed and dispersed in a Pasteur pipet containing 0.5 ml of saline. Then the cells and medium were separated by recentrifugation at 2000 x g for 30 min.

The packed cells were lysed by the addition of 0.1 ml of 10% Triton and 0.3 ml of 0.15 M NaCl. Cell lysates were individually applied with Pasteur pipets to filter paper disks (12). The disks were dried with a hair dryer, placed in a beaker of cold 10% trichloracetic acid (TCA), and washed by the method of Robinson and Harris (13). A 15 min, 37 C incubation with 95% ethanol-ether-chloroform (2:2:1) was added just prior to the ethanol-acetone (1:1) washes to ensure further the removal of lipids. Washed disks were dried, placed in vials containing 10 ml of scintillation fluid (0.5% PPO, 0.01% POPOP in toluene), and counted in a liquid scintillation spectrometer that had an efficiency of 58%.

Isolation of Labeled lDL by Ultracentrifugal Flotation

For each sample an aliquot of medium with carrier was adjusted to d 1063; 1 ml of rat serum at d 1.063 was added; and the resultant solution was centrifuged at 100,000 x g for 16-18 hr. The top 3 ml was removed by tube slicing and placed in another cellulose acetate tube; 1 ml of rat serum at d 1.063 was added and the tube was filled with 1.6 M NaCl solution (d 1.063), and recentrifuged as before. The top 1.5 ml after the second centrifugation was then applied to filter disks for TCA precipitation (12).

Immunoprecipitation of Labeled lDL and Carrier lDL From Incubation Medium

Labeled lDL and carrier lDL were harvested from the medium by precipitation with an equivalent amount of specific anti-lDL antiserum. The immune precipitates were incubated at 37 C for 45 min, shaken, stored at 4 C for 15 hr, and then collected by centrifugation at 2000 x g for 30 min at 4 C. The precipitated complexes were washed twice with 0.02 M tris-0.15 M NaCl solution (pH 7.6), and allowed to stand between centrifugations at 4 C for 30 min to promote aggregation. The washed precipitates were solubilized at pH 11.0 in 0.5 ml of 0.02 M tris-1.6 M NaCl solution (14), and were individually applied to filter paper disks, washed and counted as described for cell lysates.

Immunochemical and Electrophoretic Studies

Lipoprotein immunodiffusion and immunoelectrophoresis, the latter modified by the use of agarose and an electrophoretic duration of 60 min, were carried out according to the method of Levy and Fredrickson (15). The gels

were then stained for lipid with oil red O. Electrophoresis of rat serum lipoproteins was carried out on cellulose acetate strips by the method of Briere and Mull (16) and the strips were stained for protein with Ponceau S dye.

RESULTS

Effect of Cofactors

In contrast to the absolute requirement of added cofactors for acetate incorporation into cellular lipids (D.M. Capuzzi and S. Margolis, submitted to Lipids, and 17), leucine was incorporated into cellular protein in unfortified buffers. However, as indicated in Table I, the addition of Mg^{++}, nicotinamide, phosphate and succinate produced a 3 1/2-fold stimulation of leucine incorporation. Each cofactor contributed a significant stimulation of leucine incorporation when added individually, but Mg^{++} was clearly the most important requirement for this enhancement. All four cofactors were routinely used in studies of leucine incorporation. The effect of Mg^{++} on leucine incorporation was tested at concentrations that varied from 2.5 mM to 10 mM, the optimal range for ribosomal protein synthesis (18). The Mg^{++}-induced stimulation of leucine incorporation peaked at a concentration of 7.5 mM.

A number of cofactors that are necessary for protein synthesis in liver homogenates (18) were tested for their effects on leucine incorporation in liver cells. The results in Table II indicate that the addition of ATP, ATP + GTP, and an unlabeled amino acid mixture further stimulated leucine incorporation. But the pH 5 enzymes and ATP-generating system were mildly inhibitory to this process. Moreover, ATP caused rapid clumping of cells and inhibition of acetate incorporation into cellular lipids (D.M. Capuzzi and S. Margolis, submitted to Lipids), and these cofactors were not routinely included in subsequent incubations.

TABLE II

Effects on Cellular ^{14}C-leucine Incorporation of Cofactors Required for Protein Synthesis in Liver Homogenates[a]

Additions	Relative ^{14}C-leucine incorporation, %
None	100
ATP	133
ATP, GTP	140
ATP, GTP, ATP-generating system[b]	97
ATP, GTP, ATP-generating system, pH 5 enzymes	77
Unlabeled amino acid mixture[c]	131

[a]Incubation conditions were as described for combined cofactors in Table I. Where indicated, additions were made to give the following final concentrations: ATP (1.2 mM); GTP (0.1 mM); phosphoenolpyruvate (6.4 mM); pyruvic kinase (0.05 mg/ml); pH 5 enzymes (0.6 mg/ml). Results are based on three experiments.

[b]Phosphoenolpyruvate and pyruvic kinase.

[c]The amino acid mixture contained the following L-amino acids in a final concentration of 0.025 mM each: alanine, glycine, methionine, cysteine, threonine, serine, valine, isoleucine, aspartic acid, glutamic acid, proline, histidine, lysine, arginine, phenylalanine, tyrosine, and tryptophan.

Incubation Media

The capacity of various incubation media to support protein synthesis depended on whether

TABLE III

Effect of Incubation Media on ^{14}C-leucine Incorporation Into Cellular Protein[a]

Medium	Relative incorporation of ^{14}C-leucine, %	
	Cofactors absent	Cofactors present
Tris-KCl	100 (830 cpm)	100 (2250 cpm)
Tris-NaCl	99	70
Tris-sucrose	57	68
Tris (0.001 M)-sucrose (0.06 M)	33	73
Ringer's lactate	204	106
Locke's citrate[b]	109	21
Sodium phosphate (0.1 M)	86	56
Rat serum	65	15

[a]Incubation conditions were as described in Table I except for substitution of the indicated buffers for tris-KCl. Comparison of relative effects of medium composition on leucine incorporation were made in the presence and absence of the combined cofactors listed in Table I. All media were at pH 7.3 (37 C). The top three media listed were 0.02 M in tris and 0.1 M in the other component.

[b]Contains, per liter, 9.5 g Na Cl, 0.075g KCl, 0.150 g $NaHCO_3$, 1.0 g glucose and 7.9 g sodium citrate.

TABLE IV

Comparison of Methods of IDL Isolation[a]

Experiment	^{14}C-leucine incorporation into IDL[b], cpm	Isolation method
1	124	Flotation
	146	Immune precipitation
2	89	Immune precipitation
	82	"Cleared" immune precipitation[c]

[a]Each flask contained 4 to 6 x 10^6 cells, ^{14}C-leucine (3.75 μc), tris-HCl (20 mM), pH 7.3 (37 C), KCl (100 mM), $MgCl_2$ (7.5 mM), potassium phosphate (10 mM), nicotinamide (7.5 mM), sodium succinate (12 mM), penicillin (1 mg/ml), and streptomycin (0.03 mg/ml) in a total volume of 4.0 ml. The cells were incubated at 37 C in 25 ml Erlenmeyer flasks for 2 hr in a water bath shaker set at 130 cycles/min. The separation of medium from cells after incubation and the techniques for isolation of labeled IDL from the medium are described in Methods. Each value in the table represents an average of duplicate samples. For each isolation method, an additional flask which contained all components except the label, was incubated for 2 hr and placed on ice. Cold leucine, labeled leucine, and carrier IDL were then added in that order. The radioactivity in the IDL of zero time samples was subtracted from that of samples incubated with label to determine leucine incorporation into IDL.

[b]Results are expressed as counts/min per 4 x 10^6 cells.

[c]Prior to IDL assay the medium was first "cleared" with chicken ovalbumin-anti-ovalbumin.

the four cofactors were present (Table III). Cellular leucine incorporation was two times greater in Ringer's lactate solution than in any other unfortified medium tested. Solutions of tris-KCl, tris-NaCl, sodium phosphate (0.1 M), and Locke's citrate were roughly comparable in activity in the absence of cofactors.

The addition of the four cofactors substantially enhanced leucine incorporation except in the case of rat serum and Locke's citrate. The cofactor enhancement was greatest in tris-KCl medium. In fact, the rates of cellular protein synthesis in tris-KCl and Ringer's lactate media were similar after cofactor addition (Table III).

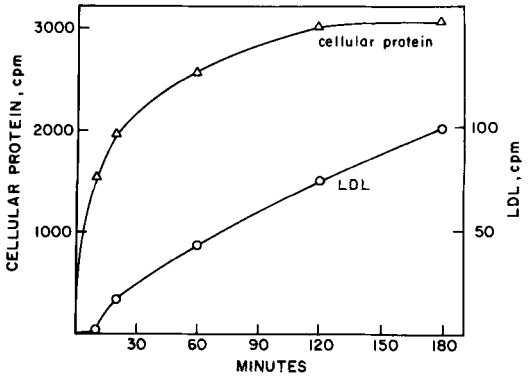

FIG. 1. Time course of leucine incorporation into cellular proteins and extracellular IDL. Incubation conditions were the same as those described under Table I.

Reliability of IDL Isolation Techniques

To study the synthesis of IDL by isolated liver cells, it was necessary to employ an assay technique specific for rat IDL. Initially, IDL was isolated by the standard technique of ultracentrifugal flotation. No contamination of the IDL with HDL was found by cellulose acetate electrophoresis. However, for technical simplicity, a rapid immunoprecipitation technique, using a rabbit antibody against IDL, was developed for specific isolation of labeled IDL from the incubation medium. The purity of the IDL antigen and anti-IDL antibody were demonstrated by immunodiffusion and immunoelectrophoresis. On immunodiffusion, a single, distinct precipitin line formed between the central anti-IDL antiserum well and peripheral wells that contained either IDL isolated at d 1.063, IDL isolated at d 1.035, or whole rat serum. No precipitin line formed between anti-IDL and rat serum proteins of $d>1.063$. The purity of anti-IDL was also confirmed by electrophoresis of IDL carrier or rat serum followed by gel diffusion against anti-IDL. In both cases a single precipitin line which occupied the entire beta region was observed. The presence of a single precipitin line in each case established the suitability of anti-IDL prepared against $d<1.035$ rat serum lipoproteins for specific isolation of labeled rat IDL. The solitary immunoprecipitin line that formed on Ouchterlony double diffusion between the IDL antigen and anti-whole rat serum demonstrated

TABLE V

Incorporation of ^{14}C-leucine Into IDL Isolated in the Absence of Carrier IDL[a]

Rabbit antiserum tested	Radioactivity in immune precipitate[b]	
	Experiment 1	Experiment 2
Anti-rat IDL	95	219
Anti-ovalbumin[c]	70	134

[a]Incubations were carried out under the conditions listed in Table I, except for the absence of IDL carrier. After removal of the cells, 0.1 ml of rabbit antiserum was incubated with the medium at 37 C for 1 hr. Then 0.1 ml of Hyland anti-rabbit gamma-globulin goat serum was added and the incubation was continued for an additional hour. The precipitates were refrigerated for 16 hr at 4 C. The precipitates were then collected by centrifugation, dissolved, applied to filter paper disks, washed, and counted as outlined in Methods. Each value represents an average of duplicate samples, obtained by subtraction of the zero time control value for each experiment.

[b]Results are expressed as total IDL cpm per sample.

[c]This antiserum was a control antiserum from rabbits that had been immunized using chicken ovalbumin antigen.

the purity of this antigen.

When labeled IDL was isolated with carrier from the medium the results shown in Table IV indicate close agreement between the values obtained by flotation and immunoprecipitation. In a refinement of the immunoprecipitin technique, chicken ovalbumin and anti-ovalbumin were first added to the medium in order to "clear" it by precipitation and removal of nonspecific labeled proteins (19). As shown in Table IV, this procedure caused no significant decline in the radioactivity recovered in IDL. The immunoprecipitation method was used in all further studies of IDL production from ^{14}C-leucine.

Time Course of Leucine Incorporation Into Liver Cells and Rat Serum IDL

The time course depicted in Figure 1 shows that ^{14}C-leucine incorporation into cellular protein was rapid at first, but gradually reached a plateau by 2 hr. In contrast, after a 10 min lag period, labeled IDL appeared in the extracellular medium and was released at a linear rate for 3 hr. The radioactivity in IDL represented 15% to 20% of the label incorporated into total medium proteins. Puromycin (2 mM) caused a 90% decline in the incorporation of label into both cellular protein and IDL.

Isolation of IDL Without Carrier

Since no visible precipitin reaction occurred without added carrier, IDL was precipitated by the addition of anti-rat IDL followed by anti-rabbit gamma globulin. Precipitates which formed when the rabbit antiserum contained anti-IDL were more highly labeled than those formed when the antiserum lacked antibodies to rat IDL (Table V). However, the omission of carrier IDL resulted in a marked decline in IDL recovery and poor agreement between duplicate samples regardless of the method of IDL isolation. Nevertheless, labeled IDL could indeed be isolated in the absence of carrier.

Effect of Hepatic Lipogenesis and Triglyceride Feeding

The following group of experiments was designed to determine whether the rate of IDL production from leucine could be changed by in vitro or in vivo manipulations. The in vitro modification was the addition of the lipogenic cofactors (D.M. Capuzzi and S. Margolis, submitted to Lipids) to the incubation medium; the in vivo modification involved short term alimentation of certain rats with a triglyceride suspension. The results in Table VI show that both manipulations augmented the rate of IDL formation. The addition of the lipid cofactors more than doubled IDL production, while cellular leucine incorporation was only 50% greater than control values. This stimulation by cofactor addition was identical in both ad lib. and triglyceride-fed animals, and suggests that IDL production was stimulated by the concomitant synthesis of new lipids. As further shown, triglyceride feeding was itself responsible for a marked increment in labeled IDL production. The increase in IDL release was observed both in the presence and absence of lipid cofactors. This effect was not attributable to a chance in amino acid pool size since there was concurrent decline in cellular protein synthesis in the triglyceride-fed animals. The results in Table VI are mean values based on experiments with 12 rats; the differences are significant at the 5% level of probability by the Student's t-test analysis.

TABLE VI

Effects of the Lipogenic Cofactors and Dietary Triglycerides on ^{14}C-leucine Incorporation Into IDL and Cellular Protein[a]

Diet	Cofactors for lipid synthesis added	Leucine incorporation[b]	
		Cellular protein, cpm	IDL, cpm[c]
Ad lib.	0	2841 ± 278	77 ± 4
	+	4153 ± 253	153 ± 29
Triglyceride fed	0	1435 ± 313	168 ± 44
	+	2070 ± 461	383 ± 130

[a]Each incubation mixture of 4 ml contained 4 to 6 x 10^6 cells, tris-HCl (1 mM), pH 6.9 (37 C), and sucrose (6 mM). Other incubation conditions are indicated in Table I. Where indicated, lipid cofactors were added as 0.15 ml of a solution containing $MgCl_2$ (100 mM), $MnCl_2$ (3 mM), sodium succinate (300 mM), Coenzyme A (1 mM), sodium citrate (100 mM), nicotinamide (200 mM), NADP (7 mM), and glucose-6-phosphate (50 mM). The ad lib.-fed group (six female rats) were maintained on Purina Laboratory Chow. The triglyceride-fed group (four female and three male rats) received 4 ml of Ediol-olive oil (1:1) by stomach tube 13 and 4 1/2 hr prior to sacrifice.

[b]Results are expressed as cpm per 4 x 10^6 cells ± the standard deviations of the means.

[c]IDL were isolated by immune precipitation.

Effect of Inhibition of Lipogenesis

Since IDL production appeared to be accelerated by the formation of new lipids, it was logical, in converse fashion, to prevent lipid synthesis and to observe the effect of this on the generation of IDL. It was found that the anti-hyperlipemic drug, clofibrate, added in vitro to dispersed liver cells, inhibited label incorporation into cellular lipid by 95%, into cellular protein by 16%, and into extracellular IDL by 9%. Thus the rate of IDL production was not significantly altered by clofibrate. Perhaps the rate of synthesis of only the VLDL fraction of IDL was responsive to alterations in the rate of lipogenesis, while IDL synthesis was unaffected.

DISCUSSION

Amino acid incorporation into proteins has been studied in hepatocytes prepared by Ca^{++} chelation (7,8,21), K^+ chelation (8,9), and by enzymatic digestion (9,20). However, scant information is available on the requirements for optimal protein synthesis in isolated liver cells. Moreover, little is known about the biosynthesis of specific proteins by these cells or about the cellular control mechanisms that govern their rate of production.

Experiments with hepatic ribosomes and microsomes that found Mg^{++} (18) and K^+ (22) essential for optimal rates of protein synthesis, may explain the importance of these ions for optimal leucine incorporation in liver cells. The presence of K^+ in Ringer's lactate and tris-KCl buffers may account for their observed effectiveness in view of the profound cellular leakage of K^+ that occurs during cell preparation (23) and the inability of isolated liver cells to accumulate K^+ from the medium (24). The stimulatory effects of nicotinamide and succinate on leucine incorporation probably reflect a beneficial effect on basic metabolic processes in the cells as described (D.M. Capuzzi and S. Margolis, submitted to Lipids).

Conflicting reports on high energy requirements (7,8) for protein synthesis undoubtedly reflect variance in the biochemical characteristics or quality of the particular hepatocyte preparation used. Friedman and Epstein (8) reported a striking stimulation of leucine incorporation by inclusion of ATP, GTP, ATP-generating system, and the pH 5 enzymes, and marked inhibition with streptomycin. However, their report does not indicate whether their crude cell preparations were washed free of subcellular debris before incorporation studies were begun. In the present study, leucine incorporation was enhanced 30-40% by the addition of ATP and GTP, but the ATP-generating system and pH 5 enzymes were inhibitory. Furthermore, there was no inhibition of leucine incorporation in the presence of penicillin and streptomycin even when antibiotic concentrations were increased fourfold over those that we routinely employed. The brisk rate of protein synthesis during the first hour of incubation observed in the present work was consistent with the results of other workers (7,8,21). In addition, the "cell concentration effect," originally described by Bhargava and Bhargava (21), was confirmed in our experiments, and cell concentrations of 1-1.5 x 10^6 per ml were found optimal for leucine incorporation.

Very little reported data exists on the

TABLE VII

Effect of Clofibrate on ^{14}C-acetate Incorporation Into Cellular Lipid and ^{14}C-Leucine Incorporation Into Cellular Protein

Incubation conditions	Radioactivity in products[a]		
	Lipid[b], cpm	Protein[c], cpm	IDL[c]
Control	2217 (100%)	2168 (100%)	98 (100%)
Incubated with clofibrate	108 (5%)	1819 (84%)	89 (91%)

[a]Results are expressed as cpm per 4 x 10^6 cells.

[b]Cells were incubated in tris-KCl medium with 1.5 μc of 1-^{14}C-sodium acetate and the lipid cofactor mixture as described under Table VI. Incubations were carried out at 37 C for 2 hr at 130 oscillations per minute. At the end of the incubation period, the cells and medium were separated by centrifugation; the labeled cellular lipids were extracted and counted as previously described (32). Clofibrate concentration was 3 mM.

[c]Incubation conditions for ^{14}C-leucine incorporation are described in Table I. The combined cofactors were used.

formation of specific proteins by liver cell suspensions. After incubation with radioactive leucine, Friedman and Epstein (8) isolated labeled rat albumin from liver cells dispersed with sodium tetraphenylboron, but the specific activity of the albumin was much lower than that of total cellular protein. Ranhotra and Johnson (25) found that dispersed liver cells elaborated the activities of clotting factors VII-X into the incubation medium. The production of these factors was inhibited by the in vitro addition of warfarin. Enzymatically prepared hepatocytes were shown to incorporate ^{14}C-leucine into the cellular fatty acid synthetase complex (20).

The present investigation demonstrates the in vitro synthesis and release of labeled rat IDL from ^{14}C-leucine by isolated rat liver cells. Results agreed closely when this serum lipoprotein was isolated from the medium either by immunoprecipitation or by ultracentrifugal flotation. To ensure freedom of the antiserum from contaminating higher density lipoproteins (26,27), the rabbits used for preparation of anti-rat IDL were injected with (d<1.035) rat lipoproteins. The resultant antiserum produced only a single precipitin line when whole rat serum was allowed to interact with the antiserum by immunodiffusion and immunoelectrophoresis. This anti-IDL antiserum was employed for the bulk of our studies on lipoprotein synthesis. Since rat HDL does not cross-react with anti-IDL (15), immunoprecipitation by this method is a reliable means for the isolation of labeled IDL. Absence of a reduction in IDL radioactivity after application of the "clearing technique" provided additional evidence for product identity (2,19). In principle, the initial clearing precipitate should adsorb nonspecific labeled contaminants that might otherwise coprecipitate during addition of the specific antiserum.

In early experiments, ultracentrifugal flotation was employed to a limited extent for labeled IDL isolation, but the time required for repeated ultracentrifugations severely curtailed the accumulation of data. Moreover, the lipoprotein fraction, 1.035-1.063, reportedly has slight contamination with lipoprotein species other than VLDL or LDL (26,27). This contamination was probably not quantitatively significant in the present studies in view of the close agreement found using flotation at d 1.063 and immunoprecipitation with the anti-d<1.035 rat serum fraction. Other methods tested, but found unsatisfactory because of nonspecific contamination, included precipitation of IDL with dextran sulfate-Ca^{++} (28) and by sodium phosphotungstate (29).

As in most previous studies of lipoprotein synthesis, carrier IDL was added to ensure a consistent recovery of labeled IDL. Diminished recovery of labeled IDL and disparity between duplicate samples in the absence of carrier is probably due to variable adherence of the product to glassware during the transfers involved in its isolation. Nevertheless, the amount of label in IDL clearly exceeded that in control incubations. This result further defined the labeled product as IDL since there was no opportunity in this instance for binding of a labeled contaminant to carrier IDL. The addition of carrier prior to separation of cells from medium considerably improved the recovery of IDL, perhaps by displacement of labeled IDL from binding sites on the plasma membrane. Such binding sites for chylomicrons have already been demonstrated on the surface of dispersed hepatocytes (30).

The inhibition of ^{14}C-leucine incorporation into IDL by puromycin is evidence for true de novo peptide bond formation rather than mere

addition of amino acid groups to preformed peptide chains. There was an interesting correlation between the 90% reduction of ^{14}C-leucine incorporation observed with 2 mM puromycin and the 90% inhibition of the release of prelabeled lipid from liver cells incubated with 1.5 mM puromycin (31). Thus, the export of hepatic lipids required concurrent protein synthesis.

We have demonstrated, by in vitro and in vivo manipulations, certain factors which may partly control the rate of lDL synthesis. A direct relation between the rate of de novo fatty acid synthesis and the rate of release of lipoproteins of $d<1.016$ by perfused rat livers has been observed (32). Our results confirm this finding since the addition of a mixture of lipogenic cofactors to liver cells markedly stimulated the synthesis of lipid from ^{14}C-acetate and lDL from ^{14}C-leucine. These changes in the rate of synthesis of lDL were probably in large measure due to changes in the VLDL part of lDL, since VLDL is the principal glyceride carrier in the rat (33), and LDL is a minor fraction. However, because of the immunological cross-reactivity between VLDL and LDL and the consequent difficulty of separating these lipoproteins by immunochemical methods, they were isolated together as lDL. The lipogenic cofactors may prime the generation of all lipid-containing molecules by the cells or may stimulate production of lDL-peptide secondary to an increased formation of triglyceride cargo.

Dietary triglyceride may be another important determinant of the rate of lDL synthesis. Thus, the enhancement of lDL synthesis and the reduction in synthesis of cellular protein observed in cells prepared from triglyceride-fed rats may reflect increased uptake of fatty acids by the liver cells by lipolysis of chylomicron triglyceride. This interpretation gains support from the observation (34) that rat livers perfused with media containing fatty acids exhibited an acceleration of amino acid incorporation into VLDL and of VLDL release into the perfusate, but a depression of incorporation into other liver proteins.

The present data demonstrate the feasibility of using isolated liver cells for study of the synthesis of specific hepatic proteins and the factors that regulate their rate of production. These results may reflect physiological mechanisms that control lDL production since they are consistent with perfused liver data. Thus, isolated liver cells may play an important future role in elucidation of the regulatory determinants involved in cellular biosynthesis.

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Oxygenated *Trans*-3-Olefinic Acids in a *Stenachaenium* Seed Oil[1]

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ABSTRACT

Interesting differences were found in oils from two samples of *Stenachaenium macrocephalum* (Compositae) seed with dissimilar storage histories. One contained significant amounts of epoxy acids (6.5%) and hydroxy conjugated dienoic acids (5.6%), but the other contained no more than 1% of these oxygenated acids. Characterization of components in the former oil established that the principal epoxy acid (4.0%) is the previously unknown *cis*-9,10-epoxy-*trans*-3,*cis*-12-octadecadienoic acid. The conjugated dienols include two additional new acids with Δ3 unsaturation (2.5%): 9-hydroxy-*trans*-3,-*trans*-10,*cis*-12-octadecatrienoic and 13-hydroxy-*trans*-3,*cis*-9,*trans*-11-octadecatrienoic acoids. The nonoxygenated acids, except for the large amount (40%) of *trans*-3,*cis*-9,*cis*-12-octadecatrienoic, are those that commonly occur in seed oils.

INTRODUCTION

Seed oil of *Stenachaenium macrocephalum* (DC.) Benth. & Hook. f. contains as its major fatty acid *trans*-3,*cis*-9,*cis*-12-octadecatrienoic acid (1), first characterized in the seed oil of *Calea urticaefolia* (2). Our original sample of *S. macrocephalum* seed (No. 44843) from Uruguay provided oil with only 1% of material (calculated as epoxyoleic acid) capable of reacting with HBr under the conditions of the oxirane oxygen determination (3). Oil from a second Uruguayan sample, No. 47294, stored two years at 5 C before analysis, contained 12 times as much of the reactive material. Investigation of this difference in composition has led to the identification of seven oxygenated acids, four of which have been reported in other seed oils (4). The other three contain *trans*-3 unsaturation and have not been described previously.

MATERIALS AND METHODS

S. macrocephalum seed was prepared for analysis and analyzed for oil content and characteristics as previously described (5). Methyl esters were prepared from the oil by methanolysis catalyzed by sodium methoxide (6). A Perkin-Elmer IR-137 spectrophotometer was used to measure IR absorption of CS_2 solutions in 1 mm NaCl cells. UV spectra were obtained from solutions in USP absolute ethanol in 1 cm cells on a Beckman DK-2A spectrophotometer.

Gas liquid chromatography (GLC) of methyl esters was carried out in a Packard Model 7401 chromatograph equipped with two glass columns. One, 12 ft x 1/4 in., was packed with 5% LAC-2-R 446 on 60/80 mesh Chromosorb W; the other, 4 ft x 1/4 in., with 5% Apiezon L on 60/80 mesh Chromosorb W. The oven was held at 200 C. This same equipment was used to chromatograph oxidation products, but the temperature was programmed from 40 to 200 C at 7 C/min.

NMR spectra were obtained with a Varian HA-100 spectrometer. Samples were dissolved in deuteriochloroform or carbon tetrachloride, and tetramethyl silane was added as an internal standard.

Epoxy esters were ozonized in dichloromethane in a dry ice :acetone bath and the ozonides were reduced with triphenylphosphine (7,8). Esters containing hydroxyl groups were ozonized at room temperature with methanol as solvent (9). Epoxy groups were located by a modification of a method described by Maerker and Haeberer (10). In the modified procedure, 2 μl of ester, 200 μl of purified dioxane, and 40 μl of a 40% solution of periodic acid (H_5IO_6) in water were combined in a 12 ml centrifuge tube. The solution was shaken mechanically for 15 min before 1 ml water and 0.5 ml petroleum ether were added. The tube was shaken several times to extract the oxidation products into the petroleum ether layer. Fifteen-microliter portions of the petroleum ether layer were injected into the GLC for analysis.

Thin layer chromatographic (TLC) plates with Silica Gel G layers, 275 μ thick, were used for analyses. The solvent system was hexane and ether, 80/20. As a test for epoxides, picric acid was applied to TLC plates according to the method developed by Fioriti and Sims (11). Preparative TLC plates with 1 mm layers of the same absorbent were used with the developing

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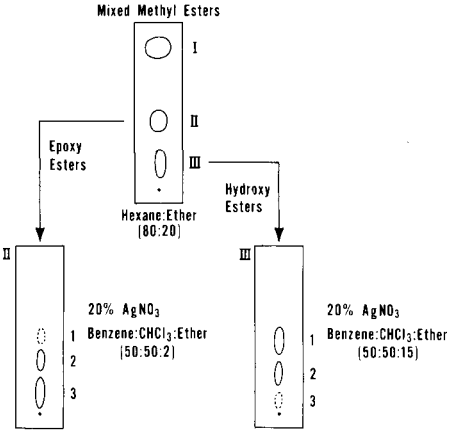

FIG. 1. TLC separation of methyl esters prepared from *Stenachaenium macrocephalum* seed oil.

solvent to fractionate the esters. Oxygenated esters were separated according to number of double bonds on 1 mm thick Silica Gel G layers impregnated with 20% silver nitrate. The solvent for separation of the epoxy-containing esters was benzene, chloroform and ether (50/50/2). Hydroxy esters were separated with the same solvents but with a 50/50/15 mixture.

A 12 mg sample of diunsaturated epoxy ester was reduced in 2 ml of ethanol with 0.01 ml of 64% hydrazine. The reaction was run at room temperature for 72 hr during which oxygen was slowly bubbled through the mixture. The reaction products were separated by preparative TLC as above for epoxy-containing esters.

RESULTS AND DISCUSSION

TLC of the methyl esters from the oil which had the greater HBr titration (No. 47294) indicated three different classes of compounds: I. nonoxygenated esters; II. esters with mobility similar to, but slightly slower than, methyl vernolate (methyl *cis*-12,13-epoxy-*cis*-9-octadecenoate) (4) and giving a positive picric acid test indicative of an epoxy functional group (11); and III. esters with the same mobility as a monohydroxy ester. These three groups of esters were isolated by preparative TLC. Class I received no additional study. Classes II and III were further separated by silver nitrate-impregnated TLC to give fractions: II-1, II-2, and II-3 and III-1, III-2, and III-3 (Fig. 1).

Esters With an Epoxy Functional Group

Monoenoic Epoxy Ester. GLC of II-2 gave a component (97%) with equivalent chain lengths (ECL) (12) of 19.0 when analyzed on an Apiezon L column and 23.5 upon analysis with a LAC-2-R 446 (Resoflex) column. These ECL's are similar to those found for methyl vernolate (12). This fraction also contained 1% of II-3.

The IR spectrum of II-2 was similar to that of known methyl vernolate; the 11.9 and 12.1 μ bands characteristic of epoxy esters were in evidence. Also, NMR spectra in $CDCl_3$ of II-2 and methyl vernolate were similar Both curves have a multiplet at 4.6 τ (13) representing two olefinic protons and a multiplet at 7.10 or 7.28 τ (13) (Table I) representing the protons on the carbon atoms of an epoxy group. The 7.10 τ is similar to the 7.11 τ found for the *cis* and distinctly different than the 7.36 τ found for the *trans* isomer of methyl 9,10-epoxystearate. The chemical shifts for the *cis* and *trans* epoxy esters are in good agreement with those reported by Aplin and Coles (14). Other bands in the spectra of II-2 are those commonly found for methyl esters.

Ozonolysis of II-2 and subsequent GLC of the reduced mixture resulted in one recognizable fragment. 6A (31%). [Number equals chain

TABLE I

Chemical Shifts of Selected Protons in NMR Analysis

Compound	Protons on epoxy group		Protons α to carboxyl group	
	CCl_4, τ	$CDCl_3$, τ	CCl_4, τ	$CDCl_3$, τ
16:1[3]	NA[a]	NA	7.09	7.01
18:3[3,9,12]	NA	NA	7.09	7.01
Methyl vernolate	ND[a]	7.13	ND	7.74
Cis-9,10-epoxy stearate	ND	7.11	ND	7.71
Trans-9,10-epoxy stearate	ND	7.28	ND	7.71
Fraction II-1	ND	7.13	ND	7.74
Fraction II-2	7.28	7.10	7.78	7.74
Fraction II-3	7.28	7.13	7.09	7.00
Fraction III-1	NA	NA	ND	7.74
Fraction III-2	NA	NA	ND	7.01

[a]Abbreviations: NA, not applicable; ND, not determined.

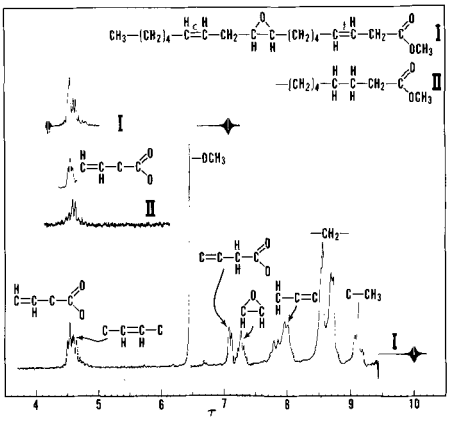

FIG. 2. NMR spectra of epoxy and *trans*-3 unsaturated esters: (I) *cis*-9,10-epoxy-*trans*-3,*cis*-12-octadecadienoate; (II) *cis*-9,10-epoxy-*cis*-12-octadecenoate; (III) olefin absorption of *trans*-3-hexadecenoate; (IV) olefin region of spectrum I after irradiation of doublet at 7.1 τ.

length of fragment; letters represent type of fragment (A, aldehyde; AE, aldehyde-ester; E, ester; EE, diester; AA, dialdehyde) (8).] Several other peaks were observed, but none with ECL's of expected ozonolysis products (8). When methyl vernolate was subjected to ozonolysis under the same conditions, only the 9AE (30%) was recognized. Presumably either ozonlysis, triphenylphosphine, or both, destroy or alter the epoxide-containing fragment. In any event, the olefinic bond in II-2 is located at the 12,13 position.

Maerker and Haeberer (10) showed that oxidative cleavage of epoxides results from reaction with periodic acid in dioxane and water. The products are aldehydic in nature. When applied to methyl oleate, this reagent did not cleave the olefinic bond. When it was applied to II-2 only two significant products, 9:1A (40%) and 9AE (35%), were observed by GLC. These products locate the epoxy group at the 9,10 position. Collectively, the chromatographic, spectroscopic, and chemical results show that II-2 is methyl *cis*-9,10-epoxy-*cis*-12-octadecenoate, the ester of an acid originally discovered in *Chrysanthemum coronarium* oil and named coronaric acid (4).

Dienoic Epoxy Ester. GLC of fraction II-3 showed a component (96%) with ECL's of 18.9 (Apiezon L) and 24.0 (Resoflex). These ECL's are consistent with those of a methyl ester having an epoxy functional group and two double bonds (12). The remaining 4% of this fraction was the epoxy-monoene (II-2).

IR absorption of II-3 included bands at 11.9 and 12.1 μ associated with the epoxy functional group. In addition to these bands, a strong band at 10.4 μ indicated the presence of isolated *trans* unsaturation equivalent to 78% methyl elaidate, as calculated by the method of Allen (15).

Ozonolysis of II-3 gave only two recognizable products, 3AE and 6A, which place the double bonds at the 3,4 and 12,13 positions. Since GLC after periodic acid oxidation revealed two major components, 9:1AE (36%) and 9:1A(53%), the epoxy functional group is on the 9,10 carbon atoms.

In deuteriochloroform, NMR of II-3 revealed a doublet at 7.00 τ (Table I) indicative of two deshielded protons on the carbon atom α to the carboxyl group (13) and confirming the presence of 3,4 unsaturation. Usually α-carbon protons in fatty acids are represented by a triplet at 7.74 τ (13). The protons on the epoxide ring produced a multiplet at 7.13 τ (2 protons) indicative of *cis* geometry. Another multiplet (Fig. 2, spectrum I), representing four olefinic protons, is centered at 4.5 τ and is derived from overlapping peaks attributable to the double bonds at the 12,13 and 3,4 positions. For comparison, the spectrum of the *cis*-12,13-olefinic protons from the compound in fraction II-2 is shown (Fig. 2, spectrum II). Similarly, the spectrum of just the 3,4, protons (Fig. 2, spectrum III is from methyl *trans*-3-hexadecenoate isolated from *Grindelia oxylepis* (16). If spin-spin decoupling is accomplished in the analysis of II-3 by irradiating the doublet at 7.00 τ (protons between the 3,4 double bond and the carboxyl group), the bands representing the 3,4 olefinic protons are converted to a singlet and the bands representing the coupled olefinic protons at the 12,13 position become evident (Fig. 2, spectrum IV). The latter bands appear very similar to the *cis* olefinic proton absorption in spectrum II, Fig. 2. IR analysis indicates only one *trans* bond, and the location of this bond was established as the 3,4 position by examination of the products of hydrazine reduction of II-3. Three products were isolated after the reduction: a saturated epoxy ester and two monoenoic esters, one of which was shown to have a *trans* bond by its IR absorption at 10.36 μ. NMR analysis of this fraction showed the presence of the double bond (multiplet at 4.5 τ) in the 3,4 position (doublet at 7.0 τ resulting from deshielded protons α to the carboxyl group). Therefore, the major component in II-3 is methyl *cis*-9,10-epoxy-*trans*-3-*cis*-12-octadecadienoate.

Saturated Epoxy Ester. GLC of II-1 showed a component with ECL's of 19.2 (Apiezon L) and 23.0 (Resoflex) which are consistent with those reported for 9,10-epoxystearate (12). Not

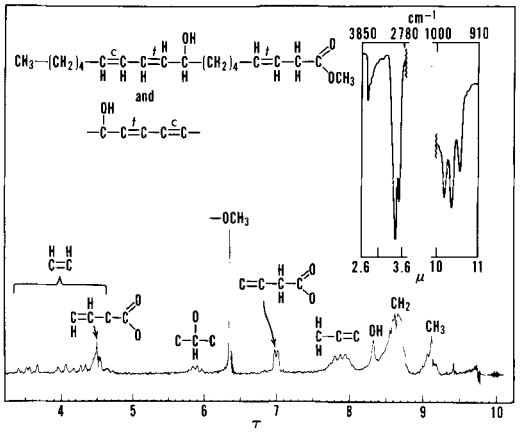

FIG. 3. NMR spectrum of hydroxy-conjugated dienes with *trans*-3-unsaturation (fraction III-2, see text and Fig. 1) with significant IR bands shown in inset.

enough sample was available for a satisfactory IR analysis. NMR analysis of II-1 in $CDCl_3$ was similar to that of methyl stearate except for a peak at 7.1 τ indicative of the protons on the *cis* epoxy group. Because oxidation with periodic acid yielded just two components, 9A (57%) and 9AE (43%), the epoxy ring must be at the 9,10 position. GLC and TLC (including positive picric acid test) analyses coupled with other results show that II-1 is methyl *cis*-9,10-epoxystearate. The occurrence of *cis*-9,10-epoxystearic acid in seed oils has previously been reported. (4).

Hydroxy-Esters

Fraction III-1. Fraction III-1 had UV absorption characteristic of fatty methyl esters with a conjugated diene functional group (λ_{max} = 233 mμ) (16) and equivalent to 74% of methyl coriolate (methyl 13-hydroxy-*cis*-9,*trans*-11-octadecadienoate, ϵ 27,063) (17). The IR spectrum of III-I was identical with that of known methyl coriolate, which shows hydroxyl absorption at 2.8 μ and *cis-trans* conjugation at 10.2 and 10.6 μ (16). NMR analysis of III-1 was the same as that reported by Tallent et al. (17). The multiple peaks representing the four olefinic protons were present in the 3.4-4.8 τ region of the spectrum. The spin-spin decoupling experiments described by Tallent et al. (17) were performed on III-1 and showed that the *trans* double bond was adjacent to the hydroxylated carbon atom as it is in coriolic acid (17).

Direct GLC of products of ozonization in methanol of an ester containing a conjugated diene system with an adjacent hydroxyl group, as in methyl dimorphecolate (18) and methyl coriolate, defined adequately the location of the hydroxyl group and the olefinic bonds (9). Ozonolysis of III-1 in this manner formed all the products that would be found from both methyl coriolate and its positional isomer, methyl α-dimorphencolate (19): 6A (20%), 6E (2%), 9AE (51%), and 9EE (13%). These results locate the hydroxy-conjugated diene system and show that two isomeric esters make

TABLE II

Stenachaenium macrocephalum Oil

Analytical data	Sample No. 44843	Sample No. 47294[a]
Oil per cent, dry basis	28	24
HBr equivalent[b]	1.0	12.3
Iodine value (Wijs)	195	187
Per cent conjugation, uv[c]	0	5.8
Fatty acid composition, %		
14:0	---	0.1
16:0	3.6	4.9
18:0	2.5	2.1
18:1	6.3	4.8
18:2	38.0	35.8
$18:3^{3,9,12}$	48.6	40.0
20:1	0.7	0.1
Cis-9,10-epoxy-18:0	---	0.6
Cis-9,10-epoxy-$18:1^{12}$	---	1.9
Cis-9,10-epoxy-$18:2^{3,12}$	---	4.0
Conjugated dienols	---	3.1
Trans-3-Conjugated dienols	---	2.5
Unknown	0.3	0.2

[a]Stored for two years at 5 C.

[b]As epoxyoleic acid.

[c]As coriolic acid.

up fraction III-1: methyl 9-hydroxy-*trans*-10, *cis*-12-octadecadienoate and methyl 13-Hydroxy-*cis*-9,*trans*-11-octadecadienoate. These are methyl esters of acids previously found in other oils (4).

Fraction III-2. UV analysis of fraction III-2 also showed strong absorption at 233 mμ, equivalent to 92% methyl coriolate. Bands at 10.2 and 10.6 μ in the IR analysis (Fig. 3, inset) showed *cis-trans* conjugation. In addition, a strong band at 10.4 μ showed the presence of isolated *trans* bonds equivalent to 105% *trans* methyl ester calculated as above (15). Absorption at 2.8 μ indicated a hydroxyl group.

NMR analysis (Fig. 3) of III-2 supported the structures indicated by IR. The peaks representing the protons on the conjugated olefinic bonds were the same as in fraction III-1. Again, spin-spin decoupling (17) showed that the system contains a hydroxyl group adjacent to a conjugated diene system with the *trans* bond in this diene system closer to the hydroxyl group. In addition, a multiple peak at 4.5 τ representing two additional protons shows the presence of an isolated double bond. The location of this bond is shown to be at the 3,4 position by a doublet at 7.0 τ (deshielded protons α to the carboxyl group) and the absence of peaks at 7.7 τ (shielded protons α to carboxyl group).

Ozonolysis of III-2 in methanol resulted in 6A (36%), 6E (4%), 3AE (10%), 3EE (4%), 6AA (7%), 6EE (2%), and 6AE (17%). These ozonolysis products would result from a mixture of the Δ3 analogs of the esters in fraction III-1, and therefore, III-2 consists of methyl 9-hydroxy-*trans*-3,*trans*-10,*cis*-12-octadecatrienoate and methyl 13-hydroxy-*trans*-3,*cis*-9,*trans*-11-octadecatrienoate.

Fraction III-3. GLC of III-3 showed one well formed peak from both the Apiezon L column (ECL = 19.7) and the Resoflex column (ECL = 27.4) while GLC of fractions III-1 and III-2 showed only broad, ill defined peaks. IR analysis of III-3 revealed a 2.8 μ band (hydroxyl) and two strong bands at 6.1 and 10.2 μ. These bands seem to be indicative of *trans*-2 unsaturation. Hopkins and Chisholm (20) reported that a strong band at 6.05 μ was associated with *trans*-2 unsaturation and S. Koritala (private communication) found the 6.1 and 10.2 μ bands from known methyl *trans*-2-octadecenoate and methyl *trans*-2-dodecenoate. No absorption indicative of conjugation was observed in the UV analyses.

NMR analysis supported the proposed Δ2 structure with peaks at 2.6-3.2, 3.9 and 4.1 τ (13). No peaks representing protons α to the carboxyl group were observed. Also, four protons at 4.7 τ show the presence of two additional double bonds which are methylene interrupted (two protons at 7.3 τ). Hydroxyl was also indicated in the NMR. Since only 1-2 mg of III-3 was collected, no rigorous characterization of this ester was attempted, but a structure such as methyl hydroxy-*trans*-2,*cis*-9,-*cis*-12-octadecatrienoate fits the special data. Whether a *trans*-2 compound was originally present in *Stenachaenium* oil or was formed during alkaline transesterification remains unknown.

Composition of *Stenachaenium macrocephalum* Oil

The ester compositions derived from both samples of *S. macrocephalum* oil (No. 44843 and 47294), as well as analytical data on the oils, are given in Table II. The percentage of total Δ3 esters (46.5%) in oil from No. 47294 is nearly the same as the percentage of $18{:}3^{3,9,12}$ (48.6%) in oil from No. 44843 that did not have a significant amount of HBr reactive material. A similar relationship is found with respect to 18:2; No. 47294 contains 40.8% of 18:2 plus oxygenated acids without Δ3 bonds while No. 44843 contsin almost an equivalent amount of 18:2 (38.0%). On the basis of these data the oxygenated acids are probably formed from the oleic, linoleic, and the $18{:}3^{3,9,12}$ acids, and only the 9,10 and 12,13 sites of unsaturation are affected.

The reason for the difference between the two samples is unknown. It may result from genetic differences or from conditions under which they were grown. The parallelism between the oxygenated acids found in Sample 47294 and those formed in sunflower seed (21,22) during storage suggest that these, too, may have been formed during storage. This possibility is strengthened because Sample 47294 was stored for two years before analysis, even though storage temperature was maintained at 5 C to minimize changes.

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SHORT COMMUNICATIONS

The Iodination of Methyl Sterculate and Deiodination of the Product to Reform the Cyclopropenoid Ring

ABSTRACT

Unlike the addition of iodine to alkenes, the radical addition of iodine to methyl sterculate has been shown to be facile at room temperature. The 1,2-diiodocyclopropanoid material produced has been deiodinated to regenerate the cyclopropenoid ring.

Fawcett and Smith (1) prepared the diiodide of methyl sterculate by the addition of bromine to sterculic acid, followed by esterification and heating under reflux with sodium iodide in acetone. Subsequent treatment of the resulting diiodide with base or zinc dust failed to reform cyclopropenoid material (as determined by IR spectroscopy) but gave conjugated and nonconjugated dienes. The diiodide was presumably formed via the dibromide because of the inherent difficulties associated with the direct iodination of alkenes.

We have shown that the addition of iodine to methyl sterculate in carbon tetrachloride or benzene solution is a facile reaction when carried out in daylight. Quantitative yields of the diiodocyclopropanoid compound are obtained at room temperature in 150 min in diffuse daylight, and in approximately 45 min when the reaction mixture is exposed to sunlight. Formation of the diiodocyclopropanoid was confirmed by vapor pressure osmometry, which gave a molecular weight of 571 ($C_{20}H_{36}O_2I_2$ = 562) and IR absorption spectroscopy [ν_{max} 1040 cm^{-1} ()]. NMR spectroscopy gave new resonances at τ8.31 ($-CH_2-\langle\rangle-CH_2-$), 8.61 [shoulder on the side of the aliphatic chain methylene resonance (*trans*-diiodocyclopropanoid (2)] and 9.05 [a small resonance attributable to the presence of the *cis*-diiodocyclopropanoid (2)]. Mass spectrometry gave ions of m/e 127 (I^+), 128 (HI^+) and 254 (I_2^+); no parent ion was observed when electron beam energies in the range of 30-70 ev were used. Differential scanning calorimetry showed that the product decomposed at 190 C with the release of iodine. Gas chromatography gave a number of peaks, probably due to the elimination of iodine and rearrangement at the operating temperature. The recovered diiodo product gave a single spot when chromatographed on thin layers of silicic acid, and was shown to be free from alkenyl or other spurious material by IR and NMR spectroscopy. Elemental analysis of the product gave: C, 42.64; H, 6.39; I, 45.01. (Calculated for $C_{20}H_{36}O_2I_2$: C, 42.73; H, 6.46; I, 45.14.)

We have also shown that it is possible to reform the cyclopropenoid ring by treatment of methyl diiodosterculate with base. Heating at 50-60 C for 36 hr with 10% potassium hydroxide in ethanol (a procedure which has been used previously to remove vicinal iodine atoms (3)), followed by acidification, resulted in the formation of 30% of the cyclopropenoid acid, as determined by NMR spectroscopy, by comparison of the area of the resonance at τ9.26 ($-C\overset{CH_2}{=\!=}C-$) with that of the acidic proton resonance. The presence of an absorption band at ν_{max} 1009 cm^{-1} in the IR spectrum of the products and a positive Halphen test confirmed the presence of cyclopropenoid material.

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Homo-gamma-linolenic Acid: A Major Polyunsaturated Fatty Acid of Swine Adrenal Cholesteryl Esters

ABSTRACT

Homo-gamma-linolenic acid is a major polyunsaturated constituent of the cholesteryl esters isolated from swine adrenals. The identity of this acid was confirmed by hydrogenation, reductive ozonolysis and a combination of thin layer and gas liquid chromatographic techniques.

A characteristic feature of rat adrenal cholesteryl esters is the high content of docosatetraenoic acid (22:4ω6) first characterized in dog adrenal lipids by Chang and Sweeley (1). The rat is unusual in this respect since the adrenals of most species contain somewhat lower concentrations of this acid and arachidonic is usually the major polyunsaturated fatty acid present (2). In the course of recent investigations on the effects of cold stress on swine, homo-gamma-linolenic acid (20:3ω6) was found to be a major polyunsaturated acid in the adrenal cholesteryl esters of this species.

Young pigs of the Yorkshire breed, five weeks of age, were maintained for six weeks on a corn-soy diet containing 10% corn oil. The animals were killed by injection of pentabarbital, the adrenals were excised, freed from perirenal fat, weighed, homogenized and extracted with chloroform-methanol (2:1 v/v) (3). After fractionation of the lipids by thin layer chromatography on silica gel G (petroleum ether-diethyl ether-acetic acid, 90:10:1), the cholesteryl esters were eluted from the gel with diethyl ether, esterified with BF_3-CH_3OH and purified as previously described (3). The esters were subjected to gas liquid chromatography (GLC) (3); the significant points of this analysis are presented in Table I.

Unlike rat adrenals, the mono- and dienoic acids accounted for the largest fraction of the unsaturated acids in the adrenal cholesteryl esters of this species. Arachidonic acid accounted for less than 10% of the total fatty acids and 22:4ω6 for less than 2%. The major acid containing more than two double bonds was the component identified as 20:3ω6, which accounted for a slightly greater proportion of the cholesteryl esters than did arachidonic acid.

To confirm the identity of this acid, an aliquot of the methyl esters was fractionated by thin layer argentation chromatography and the trienoic ester fraction isolated. The trienoic esters were subjected to GLC on a polar and nonpolar column to confirm the number of double bonds in the major component. The chain length was confirmed by hydrogenation and the location of the double bonds determined by reductive ozonolysis. These techniques have been detailed elsewhere (4). The major polyunsaturated acid in the adrenal cholesteryl esters from swine was thus shown to be homo-gamma-linolenic acid, 20:3ω6.

Although the presence of this acid as a major constituent of tissue lipids is somewhat unusual, it is not unique. The cholesteryl esters isolated from human adrenals have also been reported to contain substantial amounts of this acid (5). Unlike pig lipids, the human variety also contained substantial concentrations of 22:4ω6.

TABLE I

Fatty Acid Composition of Swine Adrenal Cholesteryl Esters

Acid	Weight %[a]
16:0	9.1±0.7
18:0	6.7±0.7
18:1	20.1±0.6
18:2	28.7±2.5
20:3ω6	11.6±1.3
20:4ω6	9.7±1.1
22:4ω6	1.1±0.3

[a]Mean of eight analyses ± SEM; minor constituents omitted from the Table.

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The Phospholipid Composition of Pig Lung Surfactant

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ABSTRACT

Surface active material (surfactant), which contained 80% lipid material, was isolated from pig lungs by the saline perfusion procedure. The lipids were analyzed by column, thin layer and paper chromatography, followed by gas liquid chromatography to determine the fatty acid composition of the major phospholipid fractions. It was found that this pig lung surfactant contained the phospholipid constituents known to be present in mammalian tissues and in addition, phosphatidylglycerol (1.5%) and lyso-bisphosphatidic acid (2.0%) were also found. These two components occurred in higher proportions in the surfactant lipids than in the lipids of the whole lung tissues.

INTRODUCTION

The alveoli of normal mammalian lungs are lined with a material which was observed by Pattle (1,2) to have high surface tension properties. This surfactant is essential for normal lung function (3-5) and in the case of infants suffering from respiratory distress syndrome, the absence of it can account for the observed cardiopulmonary abnormalities of the disease (6).

Although some workers (7-9) have described lung surfactant as a lipoprotein, others (10-12) have questioned if this material functions as part of a protein complex. In the latter case, no protein was detected in the isolated mammalian lung surfactant under examination. However in either case, the surfactant has been fully established to have a high lipid content. The major lipid component has been identified as phosphatidylcholine containing two molecules of palmitic acid (13-19).

Investigations by Body and Gray (20) on the lipid content of pig lung's surface active substance indicated the phospholipid nature of the material and also revealed the presence of two minor phospholipids which were tentatively described as phosphatidylglycerol isomers. Subsequently, these two components were isolated from whole pig lung tissue lipid extract and identified as phosphatidylglycerol and lyso-bisphosphatidic acid (21). The present communication establishes that these two phosphatidylglycerol isomers are present in pig lung surfactant and at levels higher than those in whole pig lung tissue (22,23).

METHODS

Analytical Procedures

The determination of phosphorus, aldehyde and fatty acid ester groups was carried out as previously described (23). Phosphorus at micro-level also was estimated by the method developed by Bartlett (24).

The identification of the phospholipids was carried out by the following methods. Periodate-oxidation procedures (25) detected and calculated the proportion of any nonacylated glycerol moiety within the molecular structure. Further confirmation was provided by the analyses of the intact phospholipid acetolysis products by thin layer chromatography (TLC) (26), and the mild alkaline hydrolysates, water soluble phosphate esters by paper chromatography (27). All details of these methods have been outlined earlier (21). In addition, the phospholipid components were compared with authentic standards using TLC and silicic acid impregnated paper chromatographic techniques, which have been described elsewhere (21). The fatty acid composition of the various phospholipids was determined by gas liquid chromatography (GLC) (21).

Preparation of Surfactant

Fresh, undamaged pig lungs with associated hearts were obtained from T. Wall and Sons (Meat and Handy Foods) Ltd., London. The alveolar linings were extracted within 2 hr of death by perfusing the lungs with physiological saline through the pulmonary vasculature by insertion through the heart. The principle of this perfusion technique has been described by Bondurant and Miller (28). It yielded surfactant as a stable foam. This foam was continuously washed with distilled water to eliminate salts, then frozen and lyophilized to give an off-white fine powder which was stored in vacuo at 2 C. Surface tension measurements (29) on the fresh surfactant were made with a modified Wilhelmy balance (3).

Extraction of Lipids

Freeze-dried surfactant (500 mg) was extracted once with 10 volumes of chloroform-methanol (1:1 v/v) and twice with 5 volumes of

TABLE I

Separation of Pig Lung Surfactant Phospholipids by Silicic Acid Chromatography

Eluting solvent (chloroform-methanol, v/v)	Volume, ml	Fractions collected		Per cent of phospholipid recovered	Identification (21)
		Tubes	Fractions		
49:1	200	1-25	A1	---	Neutral lipids
19:1	100	26-35	A2 } B[a]	0.5 }	Phosphatidylglycerol and
9:1	200	36-68	A3 }	4.1 }	lyso-bis-phosphatidic acid
4:1	200	69-86	A4	8.4	Phosphatidylethanolamine and phosphatidylserine
7:3	100	87-109	A5	4.8	Lysophosphatidylethanolamine and phosphatidyl inositol
5:4	360	110-161	A6	74.4	Phosphatidylcholine
3:7	400	162-188	A7	5.8	Phosphatidylcholine and sphingomyelin
Methanol	200	189→	A8	2.0	Sphingomyelin and lysophosphatidylcholine

[a]Fractions A_2 and A_3 were combined and denoted as fraction B. Details are described in the text.

chloroform-methanol (2:1 v/v). The combined extracts were washed twice with 0.2 volumes of 0.1 M potassium chloride to remove nonlipid contaminant (30). The chloroform phase was dried under reduced pressure at 40 C and the total lipid (440 mg) was dissolved in hexane. The rubber membrane dialysis technique (31) fractionated the lipids into neutral lipids (50 mg) and phospholipids (390 mg).

RESULTS

The surfactant material contained 80% (by weight) lipid of which 85% (by weight) was phospholipid. A minimal surface tension measurement of 7 dynes/cm showed the surfactant was surface active.

The phospholipid fraction (11.9 mgP) was dissolved in chloroform and applied to a chromatographic column (2 cm i.d.) of silicic acid (24 g, Mallinckrodt, A.R.). To minimize lipid oxidation (32), 4-methyl-2, 6-di-tert-butylphenol (0.005 w/v) was added to the solvents used for the preparation and elution of the column. The phospholipids were eluted from the column with chloroform-methanol mixtures and finally with methanol only, as shown in Table I. Fractions were monitored by TLC and appropriated bulked fractions (A2 + A3, denoted B) were further chromatographed into two bands (B1 and B2) by preparative TLC as described previously (21).

The resultant substances, B1 and B2, were chemically assayed by acetolysis (26). B1, the faster TLC running fraction, yielded only one glycerylacetate, namely monoacylglyceryldiacetate. On the other hand, the slower moving fraction, B2, yielded equal amounts of two major "acetates." These were identified by TLC as diacylglycerylmonoacetate and glyceryltriacetate. When mild alkaline hydrolysis (27) was applied to both fractions (B1 and B2), glycerylphosphorylglycerol was the only water soluble phosphate ester produced. However, under the periodate-oxidation reaction conditions (21,25), only fraction B2 produced formaldehyde which indicated that this fraction had two free adjacent hydroxyl groups on the intact phospholipid structure. These chemical and chromatographic properties observations confirmed that they were identical with those phospholipid components isolated during previous investigation of the total pig lung tissue lipid extract (21). Hence, B1 represented lysobis-phosphatidic acid and B2, phosphatidylglycerol. A comparison of the phospholipid composition of the pig lung surfactant with that of the whole lung tissues (21) is presented in Table II.

TABLE II

Comparison of the Phospholipid Composition of the Surfactant and Whole Lung Tissue, Expressed as a Percentage of the Total Lipid Weight

Phospholipid composition	Surfactant	Whole lung tissue[a]
Neutral lipids, %	15	60
Phospholipids, %	85	40
Cardiolipin	—	0.6
Lyso-bis-phosphatidic acid	1.5	0.3
Phosphatidylglycerol	2.0	0.3
Phosphatidylethanolamine } Phosphatidylserine }	7.1	14.4
Lysophosphatidylethanolamine	0.8	
Phosphatidylinositol	3.3	
Phosphatidylcholine	67.1	19.6
Sphingomyelin	2.7	4.8
Lysophosphatidylcholine	0.5	Trace
Plasmalogen, per cent of total lipid extract	1.2	4.8

[a]These results for total pig lung tissue lipids have been extrapolated from previous work. (21)

The fatty acid composition of the phospholipids is given in Table III. These results show that the major fatty acid associated with phosphatidylcholine is palmitic acid. The level (73.4%) is evidence of the dipalmitoyl structure of phosphatidylcholine. The other phospholipids contained an equivalent ratio of saturated to unsaturated fatty acids.

DISCUSSION

Although phosphatidylcholine was the major individual lipid component of the surfactant, the surfactant also contained substantial amounts of acidic phospholipids, namely phosphatidylglycerol and lyso-bis-phosphatidic acid. The role of these unusual phospholipids is not fully understood. It has been suggested (33) that the acidic type of bis-phosphatidic acid derivatives, with a rapid metabolic turnover, take part as carriers of the required palmitic acid for the synthesis of dipalmitoyllecithin by various enzymatic reactions (34-36). Therefore, it appears that the phosphatidylglycerol isomers do not only take part as intermediaries in the biosynthesis of cardiolipin (37-39). A comparison of the lipid composition of pig lung surfactant and total lung tissues shows that the quantity of phosphatidylglycerol and lyso-bis-phosphatidic acid in both cases are approximately proportional to the final quantity of phosphatidylcholine.

Radiochemical (40-42) and histochemical studies (43) on alveolar cells indicate that the phospholipids are synthesized by the alveolar cell wall mitochondria and transferred to the alveolar space to be incorporated with the surfactant. The physical properties of surfactant are provided by the main associated lipid, namely dipalmitoyllecithin (11). The minor constituents, particularly the acidic phospholipids, have little effect on the surface activity of the alveolar lining material. They are only secreted from the alveolar cells with their main surfactant end product, dipalmitoyllecithin. Therefore, the lipid composition of the surfactant need not correspond to that of the total lung tissues.

Conclusive studies by Watkins (44) illustrate that the surface tension properties of alveolar linings are attributed to the molecular structure of dipalmitoyllecithin. The bulkiness of the choline base resists the collapse of the molecular thin layer film during periodical compressions. This however, only applies if saturated fatty acids are combined with the polar end group since they have linear structures and fit closely together in the monolayer film. Minor phospholipids containing 50% unsaturated fatty acids could not take part in the surface active alveolar linings because these unsaturated fatty acids are nonlinear in structure.

Earlier investigations on the phospholipid composition of the mammalian lung alveolar linings have not revealed the presence of either phosphatidylglycerol isomers. Phosphatidyldimethylethanolamine, in contrast, was identified and isolated from dog lung tissues and alveolar surfactants (18). It has been suggested (21) that this latter phospholipid appearance might only apply to particular species. However, further studies (42,45-47) have also reported that

TABLE III

Fatty Acid Composition of Pig Lung Surfactant Phospholipids Expressed as Percentage of Total Methyl Esters by GLC

Fatty acid[b]	Phospholipids[a]			
	PG	LBPA	PE	PC
12:0	0.3	0.2		
13:0	Trace[c]			
14:0	2.5	2.3	1.5	5.4
15:0	0.3	0.7		0.7
16:0 br	Trace	0.5		
16:0	39.5	42.5	25.7	73.4
16:1	4.8	3.6	2.7	6.3
17:0	0.8	0.8	0.7	Trace
17:1	0.4	0.2	0.5	Trace
18:0	8.5	11.8	27.1	3.0
18:1	37.8	30.4	22.4	10.4
18:2	4.2	4.9	7.6	0.8
20:4	0.9	2.1	11.6	
Ratio, saturated: unsaturated	1:1	1:1	1:1	5:1

[a]Abbreviatons: PG, phosphatidylglycerol; LBPA, lyso-bis-phosphatidic acid; PE, phosphatidylethanolamine; PC, phosphatidylcholine; br, branched-chain.

[b]Designations: First number, chain length; second number, number of double bonds.

[c]Trace, less than 0.1%.

phosphatidyldimethylethanolamine is present in some other mammalian lung tissues. Careful examination of these latter statements suggest these workers could have been slightly misled. Under the TLC conditions they employed, phosphatidyldimethylethanolamine would have similar chromatographic properties to phosphatidylglycerol (33). They did not undertake any chemical analyses of this unusual compound.

In support of the present work, Baxter et al. (48), who thoroughly analyzed the lipid content of the total lung tissues of five different species, did not detect phosphatidyldimethylethanolamine but did conform the presence of both phosphatidylglycerol isomers. In addition, Abrams and Wigglesworth (49) also gave evidence for the occurrence of phosphatidylglycerol isomers in the lipid participant of rabbit lung surfactant material under investigation.

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The Component Triglycerides of Palm-Kernel Oil

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ABSTRACT

Triglycerides of palm-kernel oil were fractionated by gas liquid chromatography into 13 groups based on their carbon number of 30 to 54. These groups represent 99.9% of the total glyceride content. The proportion of triglyceride types in each group was calculated from the component fatty acids. These groups are defined by the nature of their constituent fatty acids but the position of the acids on glycerol is unknown. These 87 types, 24 of which are given in detail, were found. The two major components are trilaurin (19.8%) and dilauromyristin (14.1%). Only 18 types, occurring in an amount greater than 1%, together represent 80% of the total glycerides. Comparison of the glyceride content with that of coconut oil revealed many similarities between the two oils.

INTRODUCTION

The glyceride content of palm-kernel oil was first studied by Bömer and Schneider (1), Collin and Hilditch (2), and Dale and Meara (3) using fractional crystallization. Dale and Meara succeeded in isolating 10 fractions and by determining the component fatty acids deduced a possible triglyceride composition of palm-kernel oil.

As shown in a previous paper for coconut oil (4), gas liquid chromatography (GLC) fractionation of triglycerides (5) and determination of the component fatty acids of the fractions obtained, give more accurate results than the method of fractional crystallization.

In the present paper, GLC fractionation is applied to palm-kernel oil.

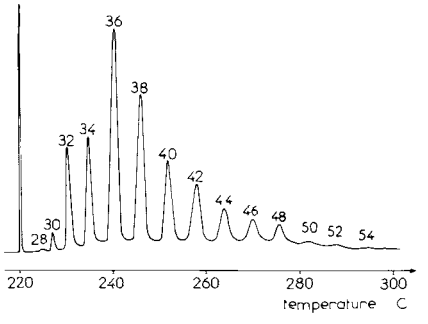

FIG. 1. GLC elution pattern of palm-kernel oil triglycerides. The triglycerides are denoted by their carbon number (total acyl carbon atoms). Operating conditions: 1/8 in. x 2.5 ft stainless steel column (JXR on 100/120 mesh Gas-Chrom Q) programmed at 3 C/min from 220 C to 320 C. Other conditions as reported in Experimental Procedures.

EXPERIMENTAL PROCEDURES

Materials

The refined palm-kernel oil was provided by the Astra-Calve Society.

The triglyceride fraction was isolated by silicic acid column chromatography (6) before GLC fractionation.

Method

Fractionation of Triglycerides. Collection of triglyceride peaks was performed according to the technique referred to above (5). A Girdel 75CD/PT chromatograph was used with a Packard modified gas fraction collector (model 852) and with a Girdel post-column stream splitter (Split ratio, 1:6). Analyses were carried out on a single stainless steel column (3/16 in. x 5 ft) packed with 100/120 mesh Gas-Chrom Q (Applied Science Laboratories) coated with JXR (Applied Science Laboratories) as described by Horning et al. (7). Since the proportion of phase is less than 1% it cannot be determined by experiment; however coating may be reproduced to a sufficient degree of accuracy under the following experimental conditions: JXR (0.8 g) was dissolved in 400 ml of methylene chloride (CH_2Cl_2); 25 g 100/120 mesh Gas-Chrom Q were added. The mixture was left to stand for 1 min; it was then submitted to progressive vacuum for 3 min. Again, it was left to stand for 1 min and was then filtered through fritted glass.

Analyses were made on quantities of 1 to 2 mg. Operating conditions were as follows: linear temperature programming from 240 C to 350 C at a rate of 5 C/min; nitrogen flow, 90 ml/min; injector temperature, 375 C; collector temperature, 350 C.

Analysis of Triglycerides. Triglycerides of palm-kernel oil were analyzed and the purity of collected peaks monitored by the previously described method (5,8). A Girdel chromatograph was used with dual flame ionization detector and with two stainless steel columns (1/8 in. x 2.5 ft) packed with JXR

TABLE I

Fatty Acid Composition of Triglyceride Groups 30 to 54 of Palm-Kernel Oil

Fatty acid composition, mole, %	Triglyceride groups[a] (mole %[b])												Total triglycerides	
	30, (1.3)	32, (8.6)	34, (9.8)	36, (25.7)	38, (16.8)	40, (9.5)	42, (7.8)	44, (5.8)	46, (4.0)	48, (4.4)	50, (2.2)	52, (2.2)	54, (1.8)	Experimental	Calculated[e]
6:0	16.9	2.5	1.2	0.6	0.2	0.6	0.5							0.5	0.8
8:0	12.0	25.6	12.8	2.7	2.3	1.7	2.1	3.3	0.4					6.1	5.2
10:0	9.8	4.2	15.3	4.3	1.9	2.8	1.3	1.2	1.9					4.9	4.0
12:0	40.3	56.9	47.2	81.3	58.9	39.1	39.8	24.3	21.3	21.3	3.8	2.7	2.1	52.1	51.0
14:0	5.8	5.1	16.2	7.1	29.3	29.9	15.2	22.1	12.5	9.3	16.5	3.7	3.0	15.7	15.6
16:0	5.6	2.4	2.8	2.5	3.2	16.1	14.9	11.9	26.0	13.0	23.7	30.1	9.9	6.3	7.9
16:1	2.4	0.8	0.8	0.2	0.4	1.7	1.7	1.1	1.5	1.2	2.9	2.9	4.2	Trace	0.9
18:0	1.5	0.5	0.7	0.3	1.0	1.8	5.6	5.7	4.2	6.5	6.6	7.1	8.0	1.8	2.2
18:1	2.2	1.1	1.6	0.8	2.4	5.3	16.4	27.7	28.0	43.0	37.9	43.7	56.5	10.2	10.1
18:2	2.0	0.4	1.0	0.1	0.3	0.6	2.1	2.5	3.5	4.4	6.0	7.2	9.6	1.5	1.4
20:0									0.4	1.0	1.6	1.2	2.5	0.2	0.1
18:3	1.5	0.5	0.4	0.1	0.1	0.4	0.4	0.2	0.3	0.3	1.0	1.4	0.9	0.2	0.4
22:0											Trace	Trace	0.4	0.5	0.1
24:0													2.9	Trace	Trace
Calculated carbon number[c]	33.5	33.4	35.4	36.3	38.4	40.9	42.9	44.9	46.4	48.2	49.8	50.5	53.1		
Difference, %[d]	+11.7	+4.2	+4.1	+0.8	+1.1	+2.2	+2.1	+2.1	+0.9	+0.4	-0.3	-2.8	-1.6		

[a]Triglycerides with the same carbon number (number of carbon atoms of the fatty acid moiety) corresponding to the peaks as they appear on the chromatogram (Fig. 1).

[b]Traces of groups 26 and 28 (0.1 mole per cent) were found.

[c]Calculated from the fatty acid composition of groups 30 to 54.

[d]$100 \times \frac{\text{calculated carbon number - true carbon number}}{\text{true carbon number}}$

[e]Calculated from the fatty acid composition of each group and from the group composition of palm-kernel oil.

TABLE II

Determination of Component Triglyceride Types of Group 42[a]

Fatty acid composition of group 42, mole %			Determination of triglyceride type composition of group 42			
Fatty acids	Experimental	Calculated[c]	Triglyceride types	Mole %	Equation system[d]	Solution
6:0	0.5	1.0	6, 18, 18	a	a=0.5x3	a = 3
8.0	2.1	4.0	8, 16, 18	b	b=2.1x3	b = 12
10:0	1.3	2.0	10, 14, 18	c	c+d=1.3x3	c = 4
12:0	39.8	39.7	10, 16, 16	d	2e+f=39.8x3	d = 2
14:0	15.2	15.0	12, 12, 18	e	c+f+3g=15.2x3	e = 42
C16[b]	16.6	17.0	12, 14, 16	f	b+2d+f=16.6x3	f = 35
C18[b]	24.5	21.3	14, 14, 14	g	2a+b+c+e=24.5x3	g = 2

[a]For definitions of groups and types see results section.

[b]Fatty acids with the same number of carbon atoms. C16 comprises mainly 16:0 and 18:1 represents the major component of C18.

[c]Calculated from the component triglyceride types as determined in the last four columns.

[d]These equations were derived by taking the fatty acid composition of group 42 (whose triglyceride composition is a, b. . .g) to be identical to the experimentally determined fatty acid composition. Number 3 figures in the second member because 100 moles of triglycerides include 300 moles of fatty acids.

on 100/120 mesh Gas-Chrom Q. Coating conditions were as follows: 1.5 g JXR was dissolved in 400 ml CH_2Cl_2; 25 g Gas-Chrom Q were added; the mixture was gently shaken without vacuum for 1 min, with progressive vacuum for 3 min, and without vacuum for 1 min, then filtered through fritted glass.

Analyses were performed with linear temperature programming from 220 C to 320 C at 3 C/min, with a nitrogen flow rate of 40 ml/min. Injector and detector temperatures were 350 C.

Under these conditions the correction factor for peak 54 was 2.32 (the factor related to peak 30 being taken as 1.00).

Analysis of Fatty Acids. The component fatty acids of the collected peaks were determined by GLC of their butyl esters (8,9); these esters were prepared in sealed tubes (internal volume, 0.5 to 1 ml) to prevent loss of short chain fatty acids.

The butyl esters were analyzed in a Girdel chromatograph with dual flame ionization detector and two stainless steel columns (1/8 in. x 4 ft) packed with 20% DEGS (Applied Science Laboratories) on acid-washed 80/100 mesh Chromosorb W (Johns-Manville). Operating conditions were: linear temperature programming from 100 to 190 C at 3 C/min; nitrogen flow, 25 ml/min; injector and detector temperature, 200 C.

RESULTS AND DISCUSSION

Palm-Kernel Oil Triglycerides

Figure 1 shows a characteristic GLC elution pattern recorded during analysis of palm-kernel oil triglycerides. Each peak groups together those triglycerides having the same number of carbon atoms and is referred to by its carbon number (total acyl carbon atoms). The triglyceride group composition of the oil as determined from the chromatogram is reported in Table I (line two). Fourteen peaks (or groups) appear on the chromatogram. Peak 36 (25.7%) and peak 38 (16.8%) are the major components and six peaks (32 to 42) together represent 78.2% of the total triglycerides. Trace amounts of peak 26 and peak 28 (0.1%) were found.

Fatty Acid Composition of the Fractionated Peaks

The 14 peaks (28 to 54) which appear on the chromatogram were collected and their purity controlled by rechromatography. The contamination of peak 28 was very high (more than 50%) and too little material was available for purification to be possible. The purity of the other peaks ranged between 92% (peak 30) and 99% (peak 32). These 13 peaks (30 to 54), together representing 99.9% of the total, were analyzed for component fatty acids without further purification.

The fatty acid composition of these peaks is reported in Table I. Under each peak the carbon number calculated from the fatty acid composition can be compared with the theoretical carbon number; the differences are expressed as percentages. The last two columns give respectively the experimentally determined fatty acid composition of the total triglycerides and that calculated from the fatty acid composition of peaks 30 to 54.

There is a close correlation between the calculated and theoretical carbon numbers (except for peak 30) and between the calculated

TABLE III

Component Triglyceride Groups and Types in Palm-Kernel Oil[a]

Groups	Moles into the oil[b]	Types	Moles % into the groups[c]	Moles % into the oil[d]
30	1.3	6, 12, 12	50	0.6
		8, 10, 12	20	0.3
32	8.6	6, 12, 14	11	0.9
		8, 12, 12	75	6.4
34	9.8	8, 12, 14	37	3.6
		10, 12, 12	48	4.7
36	25.7	12, 12, 12	77	19.8
38	16.8	12, 12, 14	84	14.1
40	9.5	12, 12, 16	37	3.5
		12, 14, 14	39	3.6
42	7.8	8, 16, 18	12	0.9
		12, 12, 18	42	3.3
		12, 14, 16	35	2.7
44	5.8	8, 18, 18	17	1.0
		12, 14, 18	60	3.5
		12, 16, 16	14	0.8
46	4.0	12, 16, 18	68	2.7
		14, 14, 18	16	0.6
48	4.4	12, 18, 18	65	2.9
		14, 16, 18	28	1.2
50	2.2	14, 18, 18	55	1.2
		16, 16, 18	39	0.9
52	2.2	16, 18, 18	86	1.9
54	1.8	18, 18, 18	70	1.3

[a]Groups: Triglycerides with the same carbon number (number of carbon atoms of the fatty acid moiety) corresponding to the peaks as they appear on the chromatogram (Fig. 1). Types: Triglycerides defined by the three constituent fatty acids considering only their chain length; fatty acid position in the molecule is unknown. Only those triglyceride types representing more than 10% of their group are given.

[b]Traces of groups 26 and 28 (0.1 mole per cent) were found.

[c]As determined according to the method reported in Table II.

[d]As calculated from the triglyceride type composition of groups and from the triglyceride group composition of palm-kernel oil.

and experimental fatty acid compositions of total triglycerides (notably for the major components, 12:0, 14:0 and 18:1).

As anticipated, the short chain fatty acids (6:0 to 10:0) are found in the low molecular weight peaks 30, 32 and 34 and the long chain fatty acids (16:0 and 18:1) in the high molecular weight peaks 40 to 54. Medium chain fatty acids (12:0 and 14:0) are present in every peak; 12:0 occurs more particularly in peaks 30 to 48 (81% in peak 36) while 14:0 is found especially in peaks 34 to 50 (30% in peak 40).

The fatty acid composition of total triglycerides determined in the palm-kernel oil under investigation accords closely with that recorded by Carsten et al. (10). There are some differences with the data obtained by Dale and Meara (3) e.g., the lauric acid content is somewhat higher (52% as compared with 45% found by these authors); this may be due to the origin of the two oils.

Component Triglyceride Types

A triglyceride type is defined here by its component fatty acids (degree of unsaturation is not considered); position of the acids on glycerol is unknown.

From the fatty acid composition it was possible to calculate the triglyceride type composition of each peak and consequently of palm-kernel oil.

This composition was calculated as previously described (4). An example (peak 42) is given in Table II. In these triglyceride types, C16 is mainly represented by palmitic acid, and C18 by oleic acid.

It will be noticed that if the values listed under "solution" are substituted back into the equations listed under "equation system" some discrepancy appears in equations 1, 2, 3 and 7. This is because short chain fatty acids are

underestimated, or long chain fatty acids are overestimated, or both (the calculated carbon number of peak 42 is 42.9). On the other hand we note good correlation in equations 4, 5 and 6. It follows that only the proportion formed by the main triglycerides is estimated with accuracy (12,12,18 and 12,14,16 for peak 42). Consequently we have considered only those triglyceride types which form more than 10% of their group. The data thus obtained are reported in Table III.

The table shows that each glyceride group contains one or, sometimes, two major components. For instance, group 30 comprises 50% caprodilaurin (6,12,12), group 32, 75% caprylodilaurin, group 34, 48% caprodilaurin (10,12,12) and 37% caprylolauromyristin, group 36, 77% trilaurin, group 38, 84% dilauromyristin. Group 40 has an almost identical content of dilauropalmitin (39%) and laurodimyristin (37%); group 42 has 42% dilauro-olein and 35% lauromyristopalmitin. Lauromyristo-olein comprises 60% of group 44, lauropalmito-olein, 68% of group 46, laurodiolein, 65% of group 48. Group 50 has a high content of myristodiolein (55%) and dipalmito-olein (39%). Palmitodiolein (86%) is the chief component glyceride of group 52 and triolein (70%) the major component of group 54.

Palm-kernel oil is seen to consist of a complex mixture of glycerides. Of the 87 types found in our investigation, only 18 types, found in amounts greater than 1%, together represent 80% of the total content, and only two of these components, namely trilaurin (20%) and dilauromyristin (14%), together form one third of the oil.

The presence of compounds containing lauric acid deserves note. Monolauroglycerides constitute one quarter, dilauroglycerides one third, and trilaurin one fifth of the glyceride content of the oil.

If we compare these results with those obtained by Dale and Meara (3) there appears one marked difference, namely, the amount of trilaurin found in the oil, 0.8% by these authors and nearly 20% by this investigation. The dilauromyristin content recorded by Dale and Meara is significantly higher (27%) than that determined here (14%). Nevertheless the proportion of fully saturated glycerides found (73%) is reasonably close to those reported by Dale and Meara (61.6%) and Collin and Hilditch (63%) (2).

Our data do not confirm the presence of monounsaturated glycerides (18%) to the extent reported by Dale and Meara (37%). The content of diunsaturated glycerides (7.4%) is significantly higher than that recorded by these authors (1.2%) but is lower than that recorded by Collin and Hilditch (12.3%). Triunsaturated glycerides (triolein) occur only in low proportion (1.3%).

It is of interest to compare the component glycerides of palm-kernel oil with those of coconut oil (4) since these oils both contain a high proportion of saturated acids of relatively low molecular weight (mainly lauric and myristic acids).

Coconut oil contains four major component glycerides, namely: caprylodilaurin, caprylolauromyristin, trilaurin and dilauromyristin. The proportion formed by each of these is in the order of 10%. Palm-kernel oil contains only two major components: trilaurin (20%) and dilauromyristin (14%). The presence in both oils of relatively high amounts of trilaurin is particularly significant. The same major component glycerides (those forming more than 1% of the oil) are present in both oils, with certain differences in proportion: caprylogly cerides (chiefly caprylodilaurin and caprylolauromyristin) are more abundant in coconut oil (21%) than in palm-kernel oil (10%), whereas the oleoglyceride content in coconut oil is lower. These differences relate to variations in the component fatty acids of the oils; the caprylic acid level in coconut oil is higher and the oleic acid level lower than in palm-kernel oil.

Nevertheless, in spite of these minor quantitative variations, the glyceride contents of palm-kernel and coconut oils show a close resemblance, as was to be anticipated from the similarity of their component fatty acids.

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Pyrolysis of Some Acetoxynonenes[1]

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ABSTRACT

Current studies on odor thresholds of hydrocarbons autoxidatively derived from fats required the preparation of 1,3- and 2,4-nonadienes. The pyrolysis of 3-acetoxy-1-nonene (I) and 4-acetoxy-2-nonene (II) was investigated as a route to 1,3- and 2,4-nonadienes, respectively. The acetates were pyrolyzed over pyrex helices at 400 C. Distilled pyrolyzates were characterized by mass, UV, IR, and NMR spectroscopy, along with gas chromatography. Since II gave approximately a 50:50 mixture of the 1,3- and 2,4-isomers, an earlier observation was confirmed that allylic esters may rearrange before elimination. Ester I gave about 85% of the 1,3-isomer and 15% of the 2,4. Presumably the 2,4-isomer arises from thermal rearrangement. In pyrolysis, both I and II give conjugated products almost exclusively. UV spectroscopy shows ϵ values in the 24,000 to 28,000 range.

INTRODUCTION

Current studies on odor threshold of hydrocarbons autoxidatively derived from fats required samples of 1,3- and 2,4-nonadienes. In 1963, 1,3-nonadiene was prepared by the Wittig reaction in 34% yield (1). The preparation of 2,4-nonadiene has never been reported.

Certain organic compounds fragment on heating to yield an olefin and other products. Pyrolyses of xanthates and tetraalkylammonium hydroxides are known as the Chugaev and Hofmann elimination reactions, respectively. A third class of compounds to undergo pyrolysis is composed of organic esters, particularly acetates and benzoates. Pyrolysis of esters has been studied extensively from both a mechanistic and synthetic aspect. But only since the advent of gas chromatography has the reaction been well understood. DePuy and King (2) reviewed pyrolysis reactions to 1960.

Although pyrolysis of saturated aliphatic acetates is well known, it has limited synthetic utility because mixtures of olefins are produced. Esters of secondary and tertiary alcohols give mixtures of olefins, whereas esters of primary alcohols give 1-olefins (3).

Rarely has the pyrolysis of unsaturated esters as a route to conjugated dienes been recorded. In 1941, Van Pelt and Wibaut (4) reported that the pyrolysis of 4-acetoxy-2-hexene gave 2,4-hexadiene as the only olefin from the reaction. Later, Greenwood (5) showed that the pyrolysis of 4-acetoxy-2-heptene gave a mixture of 1,3- and 2,4-heptadienes. He was able to isolate an ester fraction from the reaction products, which had undergone isomerization but not elimination. Similar isomerizations have been reported by Marvel and Brace (6) and by Grummitt and Mandell (7).

Although nonconjugated nonadienes have been prepared by ester pyrolysis, no references were found for conjugated nonadienes. Esters containing terminal unsaturation cannot rearrange via classical mechanisms during pyrolysis, and should offer a simple route to conjugated diolefins; however, little work has been done in this area. The report summarizes the first preparation of 1,3- and 2,4-nonadienes by pyrolysis of unsaturated acetates.

MATERIALS AND METHODS

Pyrolysis Equipment

A pyrolysis column was prepared by sealing 14/20 ground glass joints to a 375 x 12 mm Pyrex glass tube. The outside was wrapped with heating wire (10 ft of 1.2 ohms/ft), asbestos and glass tape. Column packing consisted of 1/4 in. Pyrex helices purchased from Scientific Glass Apparatus Co., Bloomfield, New Jersey. A thermocouple was placed at the middle of the tube and was monitored by a MiniMite potentiometer. Heating was accomplished by routing 110 v house current first through an ammeter and then through a large Variac. The pyrolysis column was mounted vertically and held by burette clamps.

Spectroscopic Techniques

UV spectra were recorded on a Cary spectrophotometer. The spectra were taken in spectroquality isooctane over the 200-400 nm range.

IR spectra were obtained in a carbon disul-

[1]Presented at the AOCS Meeting, Minneapolis, October 1969.

[2]North. Market. Nutr. Res. Div., ARS, USDA.

TABLE I

NMR Data for Whole Pyrolyzate From Pyrolysis of 4-Acetoxy-2-nonene

Proton	Calculated[a]	Found
α-Methyl	3.0	1.5
α-Methylene	2.0	2.5
Olefinic	4.0	4.0

[a]For 2,4-nonadiene.

fide solution on a Perkin Elmer Model 621 IR spectrophotometer.

NMR spectra were recorded on a Varian Model HA 100 spectrometer. Deuterated chloroform was the solvent and tetramethylsilane (TMS) the internal standard. Chemical shifts were measured as parts per million from TMS.

Mass spectra were taken on a Bendix Time-of-Flight mass spectrometer equipped with a heated inlet and in tandem with a gas chromatograph. The data were normalized and plotted with the aid of an 1130 IBM digital computer.

Alcohols

Non-2-ene-4-ol. *n*-Amylmagnesium bromide was prepared from 24.3 g of magnesium (1.0 mole) and 151.0 g of 1-bromopentane (1.0 mole). *trans*-2-Butenal (74 g, 1.0 mole) was dissolved in 100 ml of dry ether and added to Grignard reagent at 0 C. The intermediate was decomposed with saturated ammonium chloride, extracted with ether, washed with water until neutral, and dried over sodium sulfate. The solvent was removed under reduced pressure and the crude product distilled under reduced pressure to yield a fraction boiling at 57 C at 0.8 mm Hg. The yield was 80.0 g, 56.5% of theory. Analysis: C, 75.98; H, 12.76. Found: C, 76.88; H, 12.91.

A phthalate ester was prepared according to Airs et al. (8). The derivative was recrystallized from EtOH-CS_2 (1:1); mp 80-81 C. Analysis: C, 70.30; H, 7.64. Found: C, 70.58; H, 7.58. The neutral equivalent was 284; theory, 290.

Non-1-ene-3-ol. It was purchased from Aldrich Chemical Co. and was 99+% pure by gas liquid chromatography (GLC).

Authentic 1,3-Nonadiene. This was purchased from Chemical Samples Co. and was 97% pure.

4-Acetoxy-2-nonene. Heating unsaturated alcohols in the presence of acetic anhydride is said to cause rearrangement (9). For this reason mild reaction conditions were chosen for the preparation of acetoxynonenes. The possibility that rearrangement of the acetates occurred during synthesis must be considered. However, NMR spectroscopy showed that no rearrangement occurred during synthesis. Acetic anhydride (18.0 g, 0.18 mole) was added dropwise (at 25 C) to a solution of 25.0 g (0.18 mole) non-2-ene-4-ol and 8 ml of dry pyridine. After the mixture stood for three days at room temperature, the ester was extracted into petroleum ether, washed with dilute hydrochloric acid, and dried over sodium sulfate. Removal of solvent under reduced pressure gave 32.75 g product. GLC indicated the presence of two small unidentified impurities. Analysis: C, 71.67; H, 10.94. Found: C, 71.31; H, 11.39.

3-Acetoxy-1-nonene. To a stirred solution containing 40.0 g (0.281 mole) non-1-ene-3-ol dissolved in 100 ml chloroform, 23.0 g (0.295 mole) pyridine was added. A solution containing acetyl chloride (24.6 g, 10% excess) dissolved in 50 ml chloroform was then added dropwise at 25 C. The crude ester was purified in the manner described for 4-acetoxy-2-nonene. Despite the excess acetyl chloride, capillary GLC on DEGS revealed unreacted alcohol.

The alcohol was removed by passage through alumina as follows: Alumina (Fisher chromatographic grade 80-200 mesh) was packed into a 38 x 1.2 cm glass column fitted with a 500 ml reservoir. The crude ester (54.0 g) was dissolved in 50 ml hexane and poured on top of the column packing. Nitrogen was used to force the ester through the column until the solvent level was just to the edge of the adsorbent. Hexane (500 ml) was added to elute the ester from the column. The solvent was stripped off on a rotating evaporator, and 49 g (90.7%) of ester was recovered. Gas chromatography on a 6 ft x 1/4 in., 3% JXR column showed complete removal of unreacted alcohol. The crude ester was distilled through a 6 in. Vigreux column to give a product boiling at 57-60 C at 0.70 mm Hg. Overall yield was 78%. NMR supported the structures as 3-acetoxy-1-nonene. Analysis: C, 71.67; H, 10.86. Found: C, 70.74; H, 10.80.

Pyrolysis Reactions

Pyrolysis of 4-Acetoxy-2-nonene. 4-Acetoxy-2-nonene (13 g, 0.071 mole) was placed in a dropping funnel fitted with a joint adapted to the pyrolysis column. The ester was allowed to pass dropwise through the column maintained at 400 C. Nitrogen (30 ml/min) was used to sweep the pyrolyzate through the column. The pyrolyzate was collected in a U-trap cooled with dry ice and methyl Cellosolve. The pyrolyzate (10.0 g) was washed with bicarbonate, then with water until neutral, dried over sodium sulfate, and distilled under reduced pressure. A 3.06 g fraction boiling at 61-64 C at 3

mm was collected. Analysis: (Calculated for 2,4-nonadiene) C, 87.00; H, 13.00. Found: C, 87.11; H, 12.99.

Pyrolysis of 3-Acetoxy-1-nonene. 3-Acetoxy-1-nonene (21.14 g, 0.115 mole) was pyrolyzed at 400 C. The pyrolyzate was treated in a manner similar to the pyrolyzate from 4-acetoxy-2-nonene. Distillation of the pyrolyzate (8.67 g) at 10 mm gave 4.13 g of dienes boiling at 83-85 C.

Hydrogenation of Dienes From Pyrolysis of 4-Acetoxy-2-nonene. The pyrolyzate (0.103 g) was hydrogenated in 3 ml of ethanol with 10 mg of 10% palladium catalyst (Matheson, Coleman and Bell). The apparatus consisted of a small, magnetically stirred hydrogenation flask connected to a mercury leveling bulb manometer. The hydrogenation was conducted at room temperature for 1 hr during which the major portion of hydrogen was taken up in 15 min. Hydrogen uptake after 1 hr was 36 ml; theory, 41.8 ml corrected to STP.

RESULTS AND DISCUSSION

The dienes prepared from the pyrolysis of 4-acetoxy-2-nonene were characterized by GLC on squalane and β,β'-oxydipropionitrile. A homologous series of both conjugated and nonconjugated diolefins was chromatographed isothermally on the squalane column at 100 C to aid in identifying pyrolysis products.

Two major peaks were observed plus a smaller peak having a retention time identical to a C_9 nonconjugated diolefin. The first major peak corresponded on the log-log plot to neither a nonconjugated, nor a conjugated C_9 diolefin, nor a higher or lower homolog. Since the elemental analysis suggested a C_9H_{16} formula, a sample was hydrogenated over a palladium catalyst (Materials and Methods). Although the hydrogen uptake was slightly less than theory (36.0 vs. 41.8 ml), it clearly indicated the product's diene nature. GLC of the hydrogenated material (on squalane) gave a peak having a retention time identical to *n*-nonane, and chromatography on squalane as an admixture with *n*-nonane gave a single peak. The foregoing evidence indicated that the pyrolyzate was a mixture of isomeric nonadienes.

Mass spectrometry showed that the pyrolyzate had a molecular weight of 124, identical to that of a C_9 diolefin, but failed to corroborate the structure as 2,4-nonadiene.

The pyrolyzate from 4-acetoxy-2-nonene was analyzed by NMR spectroscopy (Table I). NMR showed four theoretical olefinic protons, a condition which establishes two double bonds in the pyrolyzate. Conclusive proof that the

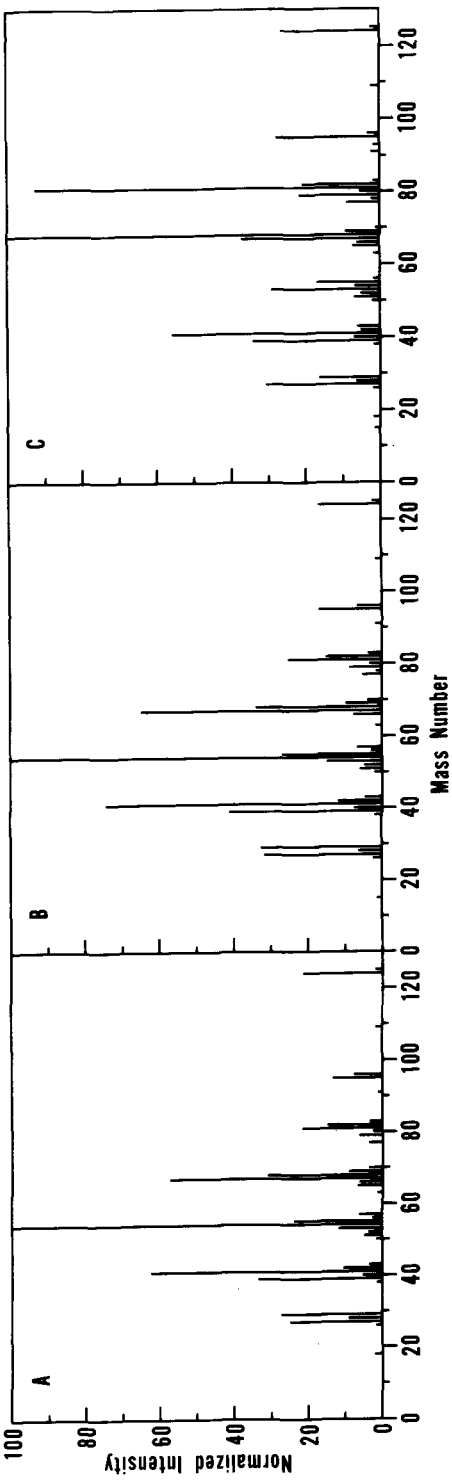

FIG. 1. Mass spectra of nonadiene isomers: (A) Authentic 1,3-nonadiene, (B) fraction 1 isolated from preparative GLC, and (C) fraction 2 isolated from preparative GLC.

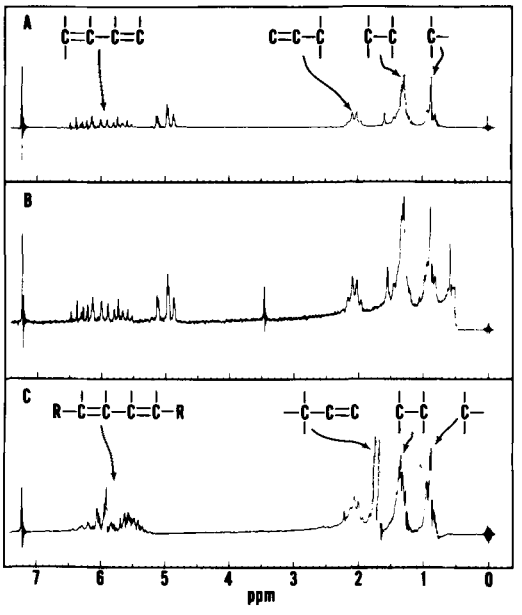

FIG. 2. NMR spectra of nonadiene isomers from pyrolysis of 4-acetoxy-2-nonene: (A) Authentic 1,3-nonadiene, (B) fraction 1 from preparative GLC column, and (C) fraction 2 from preparative GLC column.

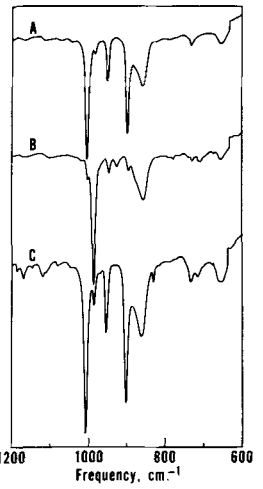

FIG. 3. IR spectra of nonadiene isomers. Solvent carbon disulfide. (A) Fraction 1 from preparative GLC column, 17.7 mg/ml, path length 0.01 cm; (B) fraction 1 from preparative GLC column, 16.0 mg/ml, path length 0.01 cm; and (C) whole pyrolyzate from 3-acetoxy-1-nonene, 21.2 mg/ml, path length 0.021 cm.

pyrolyzate was indeed a mixture of positional isomers is indicated by the number of α-methyl protons. Table I reveals that instead of the three expected (calculated as 2,4-nonadiene) α-methyl protons, only 1.5 were found. Since GLC indicated approximately a 50:50 distribution between the two major components and NMR showed only half the α-methyl protons, further evidence was obtained that the pyrolysis of 4-acetoxy-2-nonene gives a 50:50 mixture of 2,4- and 1,3-nonadienes.

To further characterize the pyrolyzate from the allylic ester, preparative GLC was investigated as a means of separation. Since the boiling point of the pyrolyzate was fairly narrow (61-63 C, 3 mm), it would appear that ordinary distillation could not be used to separate the two isomers. Separations of positional isomeric olefins by spinning band columns, however, have been reported (5). Repeated injections of 100 μl each were made until enough material was collected for analysis by NMR, IR, UV and mass spectroscopy.

Rechromatography (on squalane) of the fractions from preparative GLC showed that purities of about 90% resulted, with each fraction containing about 10% of the other isomer. In addition, GLC of the preparative GLC fractions on squalane showed that the elution order of the components was the same on both the squalane and preparative columns. Purity did, however, permit conclusive identification of the dienes.

The normalized mass spectra of the preparative GLC fractions are reproduced in Figure 1. The spectrum for an authentic sample of 1,3-nonadiene is included for comparison. The mass spectra for the preparative GLC fractions are difficult to interpret because of rearrangement peaks at m/e ratios of 54 and 68. In fraction 1 (Fig. 1, Part B) respective m/e peaks at 27 and 67 indicate cleavage occurred between carbons 2 and 3 and carbons 5 and 6. The base peak at m/e 54 is a rearrangement peak and cannot be explained by a simple carbon-carbon cleavage. Likewise, the base peak m/e 68 in fraction 2 (Fig. 1, Part C) is a rearrangement peak. If Parts A and B of Figure 1 are compared, spectra of the authentic sample of 1,3-nonadiene and fraction 1 from the preparative GLC column are identical.

In his review on mass spectrometry of organic compounds, Beynon (10) points out the difficulty of establishing the structure of simple olefins because of similarity in their mass spectra. It is, therefore, not surprising that the mass spectra of the nonadiene isomers are complex and difficult to interpret.

The NMR spectra of the preparative GLC fractions are reproduced in Figure 2, along with that of an authentic sample of 1,3-nonadiene. When the authentic sample (Part A) and fraction 1 (Part B) from the preparative GLC

column are compared, it is evident that the materials are identical. The doublet at 1.8 ppm in peak 3 (Part C) results from the olefinic methyl proton and confirms the assignment of fraction 2 as the 2,4-nonadiene isomer.

IR spectroscopy also supported the structural assignments of the preparative GLC fractions. Fraction 1 has strong bands at 898 and 1002 cm^{-1}, both characteristic of terminal (vinyl) conjugated polyenes (11) (Fig. 3). Fraction 2 has a strong band at 988 cm^{-1} indicative of *trans,trans* unsaturation. *Trans,trans* unsaturation could only result from an internal conjugated system.

The pyrolysis products from 3-acetoxy-1-nonene were analyzed by GLC on β,β'-oxydipropionitrile. Results indicate an isomer distribution of about 85% 1,3-nonadiene and 15% 2,4-nonadiene. A similar composition was obtained by GLC on squalane.

These studies indicate that pyrolysis of 3-acetoxy-1-nonene and 4-acetoxy-2-nonene gives conjugated products almost exclusively. UV spectroscopic data for the whole pyrolyzate from 4-acetoxy-2-nonene, before and after fractionation by preparative GLC, are given in Table II.

The ϵ_{max} and ϵ values are in the range reported for conjugated aliphatic polyenes (12). Data for peak 1 (1,3-nonadiene) are in good agreement with the data of Butler and Raymond (13). The whole distilled pyrolyzate from 3-acetoxy-1-nonene has a λ_{max} of 226 nm with an ϵ of 26,340 liters $mole^{-1}$ cm^{-1}.

Attempts to determine the composition of geometric isomers formed in the pyrolysis reactions has been complicated because, to our knowledge, the extinction coefficients for the pure isomers have never been reported. IR spectra (600-1200 cm^{-1}) for the preparative GLC fractions and the pyrolyzate from 3-acetoxy-1-nonene appear in Figure 3. Part A represents fraction 1 from the preparative GLC column. Strong bands at 1002 and 898 cm^{-1} due to the out-of-plane deformations of the vinyl group and a moderately strong band at 948 cm^{-1} due to the out-of-plane deformation of the *trans*-disubstituted ethylenic structure, confirm a 1,3-*trans*-nonadiene structure. Part B (fraction 2, preparative GLC column) shows weak bands at 1002 and 898 cm^{-1} due to the vinyl group resulting from the 10%, 1,3-isomer present as an impurity. A weak band at 945 cm^{-1} is due to a *trans*-disubstituted ethylenic out-of-plane band, possibly due to the trace of 1,3-*trans*-nonadiene influenced by a 2,4-*trans, cis*-nonadiene. Part C (whole distilled pyrolyzate 3-acetoxy-1-nonene) contains strong vinyl absorption bands at 1002 and 898 cm^{-1} due to the vinyl group and at 955 cm^{-1} due to the *trans* unsaturation indicating a 1,3-*trans*-nonadiene and a moderately weak band at 988 cm^{-1} due to a 2-*trans*-4-*trans*-nonadiene.

TABLE II

UV Spectroscopic Data for Whole Pyrolyzate of 4-Acetoxy-2-nonene Before and After Separation by Preparative GLC (in Isooctane)

Fraction	λ_{max} nm	ϵ $mole^{-1}$ cm^{-1}
Whole pyrolyzate	226	24,300
Peak I[a]	226	23,150
Peak II[a]	228	27,780

[a]From preparative GLC column.

The IR data show that the nonadienes from pyrolysis of 4-acetoxy-2-nonene have primarily the 1,*trans*-3 and *trans*-2,*trans*-4 structures, respectively.

These observations are in partial accord with Greenwood (5) who reported that the *trans* isomer was the major component in the 1,3-heptadiene isolated from the pyrolysis of 4-acetoxy-2-heptene. However, Greenwood found that 2,4-heptadiene was composed of a 50:50 mixture of the *trans-trans* and *cis-trans* isomers, whereas our data indicate *trans-trans* predominates.

Conditions promoting the isomerization of olefins under pyrolysis are poorly defined in the literature. Both thermal and chemical effects have been suggested as causes for isomerization of olefins during pyrolysis.

Conditions affecting isomerization during pyrolysis include acetic acid formed in the reaction and carbonization on the helices or reaction tube. Grummitt and Mandell (7) suggested acetic acid might cause isomerization when cyclic acetates are pyrolyzed. Bailey and Rosenburg (14) found that carbonization on helices and the reaction tube promoted isomerization of 1,2-dimethyl-4-cyclohexene and that carbonization occurred when more than 60-70% of the theoretical acetic acid was liberated.

Carbonization can probably be ruled out here because only trace carbon deposits were found on the glass helices or the reaction tube. Under the conditions employed, since about 47% of the theoretical acetic acid was split out, which is below the critical limit found by Bailey and Rosenburg (14), excessive acetic acid is ruled out as a cause for isomerization.

Little information is available on thermal stability of unsaturated materials to pyrolysis conditions. Bailey et al. (14,15) prepared many unusual cyclic olefins via ester pyrolysis reactions without rearrangement. 2,4-Heptadiene is

said not to rearrange at 350 C while some rearrangement (17%) of the 1,3-isomer was observed (5). Froemsdorf et al. (3) found when *cis*-2-butene was subjected to pyrolysis conditions, no conversion to the 1-isomer occurred and no *trans* isomers were formed.

Our data indicate 15% conversion from the 1,3- to the 2,4-isomer, which amount is in good agreement with Greenwood's work (5). Undoubtedly, isomerization of 1,3-nonadiene, formed during pyrolysis of 3-acetoxy-1-nonene, can be attributed to thermal effects.

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Diesters of Diols in Wheat Leaf Wax[1]

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ABSTRACT

Diesters have been isolated from the leaf wax of spring wheat, *Triticum aestivum*, L. (Selkirk variety) by chromatography. The diesters, which form 3% of the wax and which were shown by gas liquid chromatography to be a mixture of C_{51}-C_{60} esters, consist largely of *trans* 2-docosenoic and *trans* 2-tetracosenoic acid esters of 1,9-nonanediol, 1,10-decanediol, 1,11-undecanediol and 1,12-dodecanediol. The structures of the components were confirmed by synthesis.

INTRODUCTION

Small amounts of esters of *trans* 2-docosenoic and *trans* 2-tetracosenoic acids were detected in leaf wax of Little Club wheat (1) but their complete structure was not determined. Similar esters have now been isolated from leaf wax of Selkirk variety of spring wheat, *Triticum aestivum L.* and shown to consist mainly of diesters of C_9-C_{12} α,ω-diols and the above two acids.

EXPERIMENTAL PROCEDURES

NMR spectra were obtained with a Varian HA-100 spectrometer; chemical shifts are in part per million from internal tetramethylsilane and the solvent was carbon tetrachloride.

Thin Layer Chromatography

Analytical thin layer chromatography (TLC) was carried out using Silica Gel G plates, prepared with spreader setting 0.025 in (0.675 mm), the solvent was chloroform containing 1% ethanol v/v (2), and R_f values were as follows: tetracosyl tetracosanoate, 0.72; diol diester and methyl tetracosanoate, 0.55; tetracosanol, 0.15; spots were detected by spraying with 50% H_2SO_4 and heating with an IR lamp.

Ethanolysis products were examined in benzene containing 30% ethyl acetate by volume and R_f values were: ethyl *trans* 2-tetracosenoate, 0.72; tetracosanol, 0.43; 1,10-decanediol 0.04.

Preparative TLC was carried out on 20x20 cm plates of Silica Gel G, 1.3 mm thick, using chloroform containing 1% ethanol by volume as solvent; the rest of the procedure was as described by Allebone et al. (3) and gave quantitative recovery.

Gas Liquid Chromatography

Analytical gas liquid chromatography (GLC) was performed using an F and M model 402 gas chromatograph with flame ionization detectors, fitted with a 5 ft x 1/8 in. stainless steel column packed with 80-100 mesh, acid washed and silanized, Chromosorb W coated with 2% silicone SE 30. The temperature was programmed, at 2 C/min, from 300 to 380 C for analysis of diol diesters, from 100 to 160 C for diol diacetates and from 125 to 225 C for ethyl esters; the flow rate was 40 ml helium/min.

Preparative GLC was carried out with a unit of conventional design with thermal conductivity detectors fitted with a 3 ft x 1/4 in. stainless steel column packed with 10% silicone SE-30 on 60-80 mesh Anachrom ABS; the flow rate was 20 ml helium/min.

Isolation of Diol Diesters

Leaf wax (10.5 g), isolated as previously described (1), was chromatographed on Biosil A (Bio Rad Laboratories) (200 g). When most of the free alcohols had been eluted by hexane-chloroform (17:3), a mixture (3.5 g) of free acids, hydroxy β-diketones, diol diesters, alcohols and unidentified material was eluted by hexane-chloroform, 1:1. After treatment with diazomethane, this mixture was rechromatographed on Biosil A (100 g). Methyl esters (from free acids) and alcohols were eluted first and crude diol diesters were then eluted by hexane-chloroform (17:3). Pure diesters (0.3 g) were obtained by preparative TLC. NMR: CH_3, 0.88; CH_2, 1.27; H-4 of α,β-unsaturated ester, 2.18 (multiplet); CH_2 of $-CH_2-O-COR$, 4.03 (triplet); H-2, 5.70 (doublet, J=16 cps); H-3, 6.82 (two triplets, J=16 cps). The ratio of the intensities of the last four signals was 2:2:1:1 in agreement with the foregoing assignments.

A portion of the diesters was hydrogenated in ethyl acetate over 5% palladium charcoal and the product completely recovered by extraction of the catalyst with boiling chloroform.

Ethanolysis of Diol Diesters

Diol diesters (0.21 g) were refluxed overnight in ethanol containing 5% HCl by weight (25 ml), chloroform (25 ml) was added, the mixture neutralized with Ag_2CO_3, filtered, Ag

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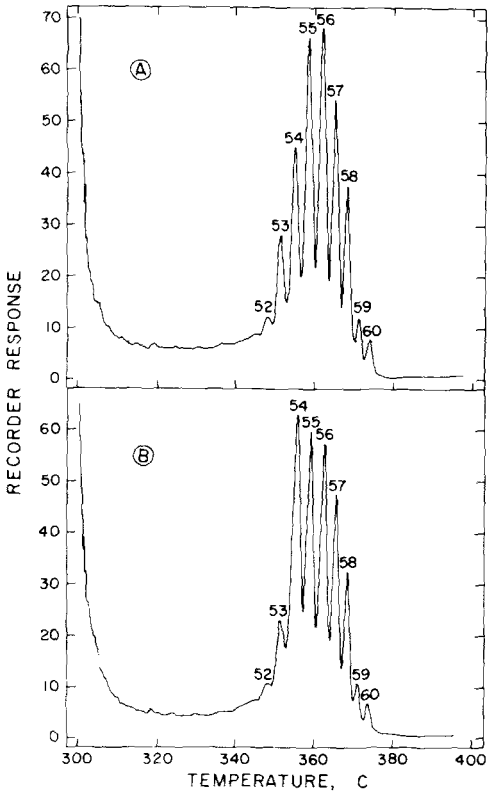

FIG. 1. GLC separation of hydrogenated diol diesters; A, before, and B, after addition of 1,10-decanediol didocosanoate.

salts washed with chloroform and the combined filtrates evaporated. TLC (benzene containing 30% ethyl acetate) showed that complete ethanolysis had occurred giving ethyl esters and diols.

The mixture was acetylated (acetic anhydride and pyridine) and chromatographed on a silicic acid column. Ethyl esters (0.15 g) were eluted with hexane-ether (97:3) and diol acetates (0.044 g) with hexane-ether (9:1). The ethyl esters were identified by GLC and NMR spectroscopy as previously described (1).

Ethyl *trans* 2-docosenoate and *trans* 2-tetracosenoate were separated by preparative GLC at 240 C, saponified and the acids crystallized from acetone. *Trans* 2-docosenoic acid had mp and mixed mp 71-72 C and *trans* 2-tetracosenoic acid had mp and mixed up 76.5-77.5 C.

Diol acetates were separated by preparative GLC at 150 C and free diols, obtained by refluxing with methanolic hydrogen chloride, were crystallized from benzene. 1,10-Decanediol had mp and mixed mp 72.5-73.5 C, 1,11-undecanediol had mp and mixed mp 60.5-61.5 C and 1,12-dodecanediol had mp and mixed mp 80.0-81.5 C. The C_9 diol was not isolated but was identified by the retention times of the free diol and the diacetate.

Synthesis of the Unsaturated Acids

Trans 2-docosenoic acid was synthesized as described by Artamonov (4) and had mp 70.5-71.5 C reference 4 gives 68.5-69 C). The methyl ester had mp 48-49 C. $C_{23}H_{44}O_2$ calculated: C 78.34, H 12.58. Found: C 78.54, H 12.64. *Trans* 2-tetracosenoic acid was synthesized by elimination of acetic acid from methyl 3-acetoxytetracosanoate (5). 3-Hydroxytetracosanoic acid (6) was converted to methyl 3-hydroxytetracosanoate which had mp 69.5-71.5 C. $C_{25}H_{50}O_3$ calculated: C 75.32, H 12.64. Found: C 75.42, H 12.51. The hydroxy ester was acetylated (acetic anhydride and pyridine) and distilled, bp/0.1 mm 200 C. A solution of acetoxy ester (2.18 g) in t-butanol (200 ml) was refluxed and a mixture of 0.1 N aqueous NaOH (99 ml) and *t*-butanol (70 ml) added slowly over 40 min. The solution was refluxed for a further 30 min, cooled, acidified and the products extracted with chloroform. The products were converted to methyl esters with methanol containing 5% HCl by weight and separated on a silicic acid column. Methyl *trans* 2-tetracosenoate (1.09 g, 58% yield) was eluted with hexane-ether 96:4 and methyl 3-hydroxytetracosanoate (0.81 g) was eluted with hexane-acetone 95:5.

Crystallization from acetone gave methyl *trans* 2-tetracosenoate with mp 53.5-54.5 C. $C_{25}H_{48}O_2$ calculated: C 78.88, H 12.71. Found: C 79.06, H 12.77.

Trans 2-tetracosenoic acid was obtained by saponification and after crystallization from acetone and had mp 76.5-77.5 C. $C_{24}H_{46}O_2$ calculated: C 78.62, H 12.65. Found: C 78.50, H 12.52.

Synthesis of Diols and C_{54} Diol Diester

Diols were obtained by reduction of the corresponding dicarboxylic acids with $LiAlH_4$ in the usual way and crystallized from benzene. The C_{10} diol had mp 72-72.5 C (lit (7) 72 C), The C_{11} diol mp 60.5-61.5 C (lit (8) 62-62.5 C) and the C_{12} diol mp 80-81 C (lit (9) 80-81 C).

1,10-Decanediol didocosanoate. 1,10-Decanediol (0.61 g) was dissolved in methylene chloride (20 ml) and pyridine (2 ml) and a solution of docosanoyl chloride (2.50 g) in methylene chloride (5 ml) added and the mixture refluxed overnight. The product was extracted with chloroform, washed with 2 N CHl and with water and crystallized from chloroform. The mp was 78.5-80 C. $C_{54}H_{106}O_4$ calculated: C 79.15, H 13.04.

TABLE I

Composition of Diol Diesters

Carbon atoms No.	Composition (by weight)	Composition calculated from random distribution
51	0.7	---
52	2.3	---
53	8.0	3.9
54	13.6	9.3
55	21.4	16.6
56	22.2	21.7
57	16.7	20.1
58	10.2	16.8
59	3.1	7.4
60	1.8	4.2

Found: C 79.11, H 13.26. This diester had the same R_f on TLC as the natural compounds.

RESULTS AND DISCUSSION

The diol diesters had unusual chromatographic properties in that they were eluted from a silicic acid column just after the major alcohol fraction but on TLC they had a much greater R_f than the alcohols making it possible to purify them by preparative TLC.

GLC analysis showed that the diesters had chain lengths ranging from C_{51} to C_{60}. The NMR spectrum contained characteristic signals at 5.70 ppm and 6.82 ppm (with coupling constants of 16 cps) due to H-2 and H-3 respectively of *trans* α,β-unsaturated esters (1). Ethanolysis of the diesters gave ethyl esters and diols, GLC analysis indicated that the ethyl esters were derivatives of *trans* 2-docosenoic and *trans* 2-tetracosenoic acids (1) and this was confirmed by isolation of the individual acids and comparison with authentic synthetic acids. The chain lengths of the diols were indicated by GLC analysis to be C_9, C_{10}, C_{11} and C_{12} and the structures were also confirmed by isolation and comparison with synthetic compounds.

The diol diesters were not completely resolved by GLC due to partial separation of saturated and unsaturated components but a fairly good separation (Fig. 1) was obtained after hydrogenation. The chain lengths of the components were established by reanalysis after addition of synthetic C_{54} diester (Fig. 1B). The composition of the hydrogenated diesters is given in Table I, the results being fairly similar to those calculated assuming a random distribution and the presence of C_{22} and C_{24} acids only.

The composition of the acids is shown in Table II and that of the diols in Table III. The acids of the diol diesters are thus limited almost entirely to the *trans* 2-unsaturated C_{22} and C_{24} acids making a surprising constrast with the acids of the monoesters and free acids of the wax which contain about 70% of saturated C_{16}-C_{30} acids in addition to the above two unsaturated acids (A.P. Tulloch and L.L. Hoffman, unpublished work). Except for the previous finding in Little Club wax (1) these acids have not been found in nature before; *trans* 2-docosenoic acid has, however, been synthesized (4).

TABLE II

Composition of Acids of Diol Diesters

Acid	Percentage (by weight)
Docosanoic	5.0
Trans 2-docosenoic	46.7
Tetracosanoic	5.2
Trans 2-tetracosenoic	40.4
Unidentified[a]	2.7

[a]Five unidentified components.

Of the diols only 1,12-dodecanediol has been reported previously in natural compounds; it occurs as an estolide in conifer waxes (10). The chain length distribution, approximately equal amounts of odd- and even-numbered components, is quite unusual since long chain natural products are usually either almost all even-numbered or all odd-numbered.

Very long chain diols have been isolated from saponified carnauba wax (11) and apple wax (12) but whether they are present in the original wax as diol diesters or as hydroxy monoesters is not clear. Diesters of long chain α,ω-1 diols are present in beeswax (2) and diesters of α,β diols in animal skin lipids (13). The diesters of 2,3-diols in wax of turkey preen gland (14) are of interest since the C_{19}-C_{23} diols consist of approximately equal amounts of even- and odd-numbered components.

Very small amounts of diesters of C_2-C_4 diols may be present in plant lipids (15) but no diesters of the medium chain length diols reported here seem to have been described previously.

ACKNOWLEDGMENTS

Experimental assistance by L.L. Hoffman, NMR

TABLE III

Composition of Diols of Diol Diesters

Diol	Percentage (by weight)
1,9-Nonanediol	13.6
1,10-Decanediol	32.4
1,11-Undecanediol	34.4
1,12-Dodecanediol	19.6

spectra by M. Mazurek and elemental analyses by W.C. Haid.

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The Turnover Time of Dietary Cholesterol in the Lactating Rat[1]

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ABSTRACT

The secretion of dietary 4-^{14}C-cholesterol into milk of the rat was determined as a function of post-feeding time by a single dose technique. The time interval which elapsed before maximum specific radioactivity was reached in milk (17-20 hr after maximum activity in the serum) suggests a route through the mammary gland involving transport of the cholesterol by intracellular membranes. It also suggests that the exogenous cholesterol is incorporated into the milk fat globule membranes rather than into the fat globules during their synthesis within the cell.

INTRODUCTION

The transport of dietary cholesterol into milk has been investigated in lactating rats by using either a steady state system of feeding to isotope equilibrium (1,2), or by a pulse system (1) where chylomicron-cholesterol uptake by the mammary gland over a very short time period (2-12 min) was examined. These experiments have demonstrated that 10-15% of the serum cholesterol is of dietary origin; the remainder is presumably synthesized de novo in the body. The relative roles of the liver and mammary gland in cholesterol biosynthesis for milk production are uncertain (1).

After feeding guinea pigs a single dose of radioactive cholesterol, the isotope was detected in milk, with a maximum activity at 2-5 days after feeding. Since the rate of uptake of the isotope into the serum was not investigated, the turnover of serum cholesterol cannot be calculated.

In this report, the single dose technique is applied to lactating rats, and the turnover time of serum cholesterol into milk fat is studied by measuring the transient levels of radioactivity in serum and milk.

EXPERIMENTAL PROCEDURES

Animals

The rats used in this study (Sprague-Dawley, 270-410 g) were each nursing six pups and were maintained on normal laboratory rat cake (0.06% cholesterol) and water (ad lib) for the duration of the experiment.

Administration of Isotope

The lactating rats were force-fed 5.0-6.2 μC (0.09-0.11 μmoles) 4-C^{14}-cholesterol (New England Nuclear, Boston, Mass.) in 0.5 ml corn oil by gavage, between 12 and 14 days post partum.

Collection and Treatment of Samples

Blood samples were collected by tail-chopping under ether anesthesia at various times after feeding. The sera were prepared by centrifugation. Milk samples were collected under slight suction after injection of 0.1 USP units of oxytocin, usually at the same time as blood samples. The material egested over the experimental period was softened with water and extracted with chloroform-methanol (2:1 v/v).

Determinations

Cholesterol levels in milk and sera were determined by the colorimetric method of Abell *et al.* (4). Radioactivity was measured by counting aliquots in Instagel scintillation fluid (Packard Instrument Co., Inc., Downers Grove, Ill.), correcting for chemical quenching by the

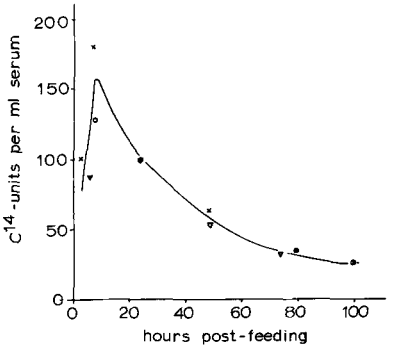

FIG. 1. Uptake of dietary 4-^{14}-C-cholesterol. Appearance of radioactivity in serum. To correct for differences in dose, uptake and the effects of dilution by intestinal synthesis of cholesterol, the radioactivity per milliter of serum is corrected to a common mean at 24 hr = 100 units/ml for each experiment. The actual values (d/m/ml at 24 hr) were 18.8 x 10^3, 9.4 x 10^3 and 10.4 x 10^3 d/m/ml, respectively. Experiment 1, x; experiment 2, 0; experiment 3, v.

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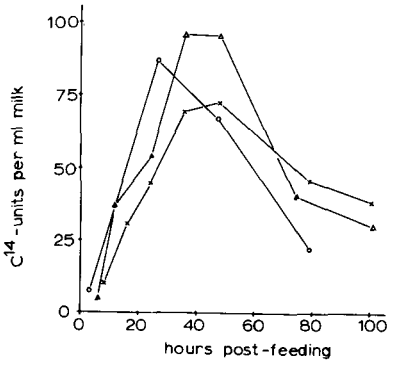

FIG. 2. Uptake of dietary 4-^{14}C-cholesterol. Appearance of radioactivity in milk. To correct for the differences in serum 4-^{14}C cholesterol levels, the same correction factors used in Figure 1 were applied to the milk radioactivity data, e.g., experiment 1, 100 units = 18.8 x 10^3 d/m. Experiment 1, x; experiment 2, 0; experiment 3, v.

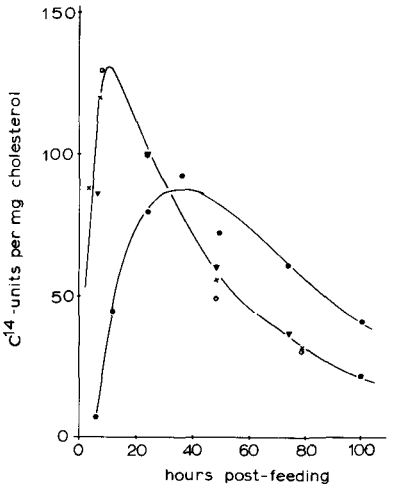

FIG. 3. Uptake of dietary 4-^{14}C-cholesterol. Comparative data on serum and milk radioactivity in experiment 3. The data are plotted as arbitrary units of ^{14}C activity per milligram cholesterol by applying correction factors as in previous Figures (experiment 3, 100 units ^{14}C = 15.3 x 10^3 d/m). Experiment 1, x; experiment 2, 0; experiment, 3 v; Milk radioactivity, experiment 3, 0.

channels-ratio method. Fecal cholesterol was assayed by counting aliquots of the extracts, correcting for color-quenching by addition of internal standard (U-C^{14}-toluene, Packard Instrument Co., Inc.) and recounting.

RESULTS AND DISCUSSION

Balance Studies

At the end of each experiment, determination of fecal radioactivity demonstrated that a high proportion of the fed isotope (77-86%) was absorbed and retained by the lactating rat for the duration of the experiment. The radioactivity recovered in the milk represented 5-11% of the total retained isotope. These results are slightly lower than those reported from isotope equilibrium studies (1,2), and probably represent the initial phase of the incorporation (i.e., serum → mammary gland → milk). Over a longer time period, the more complete system involving the reversible exchange of cholesterol between serum and other tissues (approximately 14 mg/day) (5) would involve progressively more radioactive cholesterol originally taken up by the body in general, being secreted into milk.

Radioactivity Levels in Blood and Milk

The appearance of 4-C^{14}-cholesterol in the blood stream at various times after feeding is illustrated in Figure 1. The results from three experiments are plotted to show the high degree of correlation of the response. The maximum level of radioactivity in the serum occurred approximately 8-10 hr after feeding, the level dropping after this peak with a turnover time of approximately 50 hr.

In Figure 2, the radioactivity levels in milk collected at various time intervals after feeding is given for each experiment. A fair correlation is demonstrated between the rates of secretion of 4-C^{14}-cholesterol in each animal; the maximum activity occurred 25-50 hr after feeding.

In Figure 3, the data from one experiment is examined in more detail. A direct comparison of the maximum specific activity of milk and blood cholesterol suggests that the serum contributes 70% of the milk cholesterol. However, Clarenburg and Chaikoff (1) have pointed out that dietary cholesterol in the serum is associated almost entirely with chylomicra. These workers demonstrated that the rat mammary gland rapidly takes up chylomicra-cholesterol in vivo. The specific activity of the cholesterol which the mammary gland takes up from the serum must therfore be higher than that of the serum cholesterol in general. Clarenburg and Chaikoff (1) calculate that the serum cholesterol pool used by the mammary gland has a specific activity four times greater than that of the total serum. However these authors do not correct for the effect of the liver, which would be just as likely to take up serum chylomicra, and would release the cholesterol back into the circulation as lipoprotein. Whether the mammary gland takes up lipoprotein cholesterol has not been studied, but by analogy with other tissues, the process would be expected to occur, if only at a slower rate than of chylomicron

uptake (turnover time of lipoproteins in nonlactating rats is four days) (6). The net effect of this process would be to raise the proportion of the serum cholesterol contribution to milk cholesterol.

There is evidence (7) that the liver and carcass approximate to a two-pool open system for cholesterol. If this is true, all calculations based on specific activity ratios can only be approximate, since the contribution of carcass cholesterol (nonlactating rat turnover time is 30 days, i.e., 14 mg cholesterol exchanged per day) (5) cannot be ignored.

The maximum specific activities in serum and milk cholesterol (Fig. 3) are approximately at 10 hr and 30 hr respectively. The serum value represents the slow release of 4-C^{14}-cholesterol-labeled chylomicra into the circulation, a phenomenon observed in dogs (8) and humans (9,10). The relative shapes and positioning of the specific activity curves indicates a product-precursor relationship, with a turnover time (measured as time between curve maxima) of 20 hr. The initial 4-5 hr delay in milk cholesterol secretion after feeding is indicative of the minimum time for passage of cholesterol through the mammary gland into the milk.

The turnover time cannot be determined accurately by graphical means (11) since insufficient data is available on serum cholesterol 0-10 hr, and the initial delay in milk secretion has to be known accurately and taken into account. An approximate value was calculated using a 5 hr delay and extrapolation of serum cholesterol data as 12 hr + 5 hr = 17 hr for the turnover time serum → milk.

Examination of the data by semi-log plot (Fig. 4) shows that the time between peak maxima is 20 hr, in good agreement with the results obtained above. The decline of serum and milk cholesterol specific radioactivities are exponential, with turnover times of 49 hr and 79 hr respectively.

The time required for other milk constituents to pass from the blood through the mammary gland into the milk is usually much shorter than the 17-20 hr required for serum cholesterol. For instance, data will be presented in a later publication to show that P^{32} incorporation into milk (either as inorganic phosphate or phospholipid phosphorus) is maximal 4-6 hr after intravenous injection. Similarly, serum fatty acids (either free or derived from lipid hydrolysis during entry into the mammary gland) require 6-8 hr for passage through the mammary gland to maximum activity in milk (D.J. Easter, unpublished observations).

Cholesterol is a major constituent of the membranes surrounding the milk fat globule

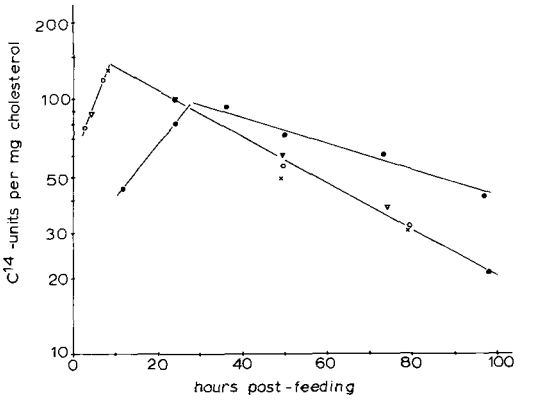

FIG. 4. Uptake of Dietary 4-^{14}C-cholesterol. Comparative data on serum and milk radioactivity in experiment 3. The data presented in Figure 3 are plotted in semi-log form. Symbols as in Figure 3.

(12). Present theories on milk secretion (13) suggest that these membranes are derived by envelopment of the globule by plasma membranes during passage of the globule out of the cell into the lumen. The plasma membrane in turn is regenerated by the process: endoplasmic reticulum → golgi vesicles → plasma membrane.

Since serum cholesterol requires 17-20 hr to pass through the mammary gland cell into milk, we must conclude that exogenous cholesterol is not incorporated into the milk fat globule during the latter's synthesis within the cell. Instead, the cholesterol is incorporated into intracellular membranes, and eventually becomes part of the plasma membrane at the apex of the cell. Exogenous cholesterol is therefore incorporated into the milk during the final stage of milk production, the enveloping of the milk fat globule by membranes during milk secretion.

ACKNOWLEDGMENTS

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Liver Phospholipids of Rats Fed a Choline-Deficient Diet Supplemented With Choline or Methionine

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ABSTRACT

The low amount of arachidonic acid in the total phospholipids in the liver of rats fed a standard type of choline-deficient diet was corrected by either choline or methionine, which also increased food intake. Choline increased the content of this fatty acid in the phosphatidyl ethanolamine but not in the phosphatidyl choline. Methionine increased both the amount of phosphatidyl choline and its content of arachidonic acid.

INTRODUCTION

Phosphatidyl choline was shown to be derived through the sequential methylation of phosphatidyl ethanolamine in the liver of choline-deficient rats (1-5). A decrease in the total amount of phosphatidyl choline was accompanied by a reduced level of arachidonic acid in phosphatidyl ethanolamine (6). It seemed possible that such phospholipid alterations might depend upon the effectiveness of the methylation of some species of phosphatidyl ethanolamine to form phosphatidyl choline rather than on a deficiency of exogenous choline itself.

Besides being deficient in choline, the test diets were also low in methionine, attained by the use of soya or peanut proteins (1-6). To ascertain if the relevant dietary inadequacy was that of methyl groups, methionine was added to a choline-deficient diet and its effectiveness in correcting the phospholipid metabolism compared to that of supplementary choline.

EXPERIMENTAL PROCEDURES

Male Wistar rats weighing 68 to 81 g, from Woodlyn Farms, Guelph, Ontario, were divided into three similar groups on the basis of body weight. One group received the choline-deficient diet previously described (6), and the other groups received this diet supplemented with either 1% choline bitartrate (General Biochemicals) or 0.5% L-methionine (Nutritional Biochemicals). After two weeks the livers were obtained from the nonfasted rats, the lipids extracted, and the total fat and lipid phosphorus determined. The phospholipids were precipitated in acetone saturated with $MgCl_2$, individual phospholipids separated by thin layer chromatography and the methyl esters of their fatty acids gas-chromatographed (6,7). To distinguish 20:5 $\omega 3$ from 20:5 $\omega 6$, relative retention times were employed (8). Methyl arachidate (Applied Science Laboratories) was used as an internal standard to quantitate the phospholipid fatty acids.

RESULTS

As shown in Table I, the addition of either choline or methionine to the choline-deficient diet improved the weight gain of rats, and methionine also significantly increased the food intake, liver weight and lipid phosphorus level. The total fat accumulated in the liver of the deficient rats was lowered to a greater extent by choline than by methionine.

The amounts of saturated and monoenoic fatty acids in the liver phospholipids were

TABLE I

Food Intake, Body and Liver Weights, Liver Lipid Phosphorus and Total Liver Lipid of Rats Fed for Two Weeks the Choline-Deficient Diet Alone or Supplemented With Choline or Methionine

Diet	Food intake, grams per two weeks	Body weight, g	Liver weight, g	Total lipid P, mg/liver	Total lipid, mg/liver
Choline-deficient	128 ± 9[a]	99 ± 6	5.75 ± 0.41	3.75 ± 0.26	919 ± 123
+ choline	143 ± 24	128 ± 4[b]	6.51 ± 0.28	4.51 ± 0.26	233 ± 9[b]
+ methionine	173 ± 9[b]	141 ± 5[b]	7.26 ± 0.33[c]	4.90 ± 0.29[c]	382 ± 30[b]

[a]Mean ± standard error of mean for seven rats.

[b]Different from the unsupplemented choline-deficient group at $P < 0.01$.

[c]Different from the unsupplemented choline-deficient group at $P < 0.05$.

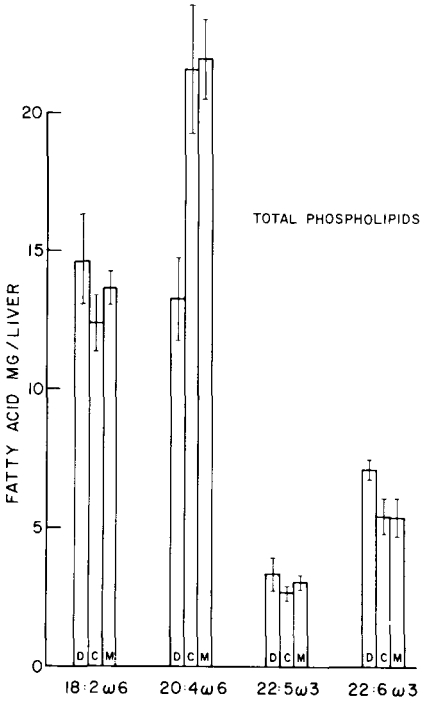

FIG. 1. Amounts of polyunsaturated fatty acids in the total phospholipids of the seven rats in each group fed the unsupplemented choline-deficient diet (D), that diet supplemented with choline (C) or with methionine (M). Values shown, means ± standard errors of the mean.

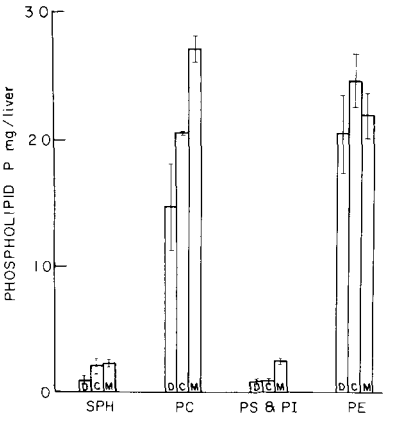

FIG. 2. Liver lipid phosphorus in sphingomyelin (SPH), phosphatidyl choline (PC), a mixture of phosphatidyl serine and phosphatidyl inositols (PS + PI) and phosphatidyl ethanolamine (PE) in three rats randomly selected from each of the groups fed the unsupplemented choline-deficient diet (D), that diet supplemented with choline (C) or with methionine (M).

similar in the three groups of rats, but, among the polyenoic acids (Fig. 1), the amount of arachidonic acid was low in the rats fed the unsupplemented choline-deficient diet. Added choline or methionine equally overcame this effect.

According to the distribution of phosphorus among the liver phospholipids (Fig. 2), the least phosphatidyl choline occurred in the rats fed the basal choline-deficient diet, and the most in those fed additional methionine. The quantities of the other phospholipids studied were not significantly altered by the dietary supplements.

The discrepancy between the amounts of fatty acids in the total phospholipids and in the combined fractions of phosphatidyl ethanolamine and phosphatidyl choline (Fig. 3) appears to indicate that the minor phospholipids were an important reservoir of linoleic acid. The amount of this fatty acid in phosphatidyl choline, like its phosphorus, was elevated by choline and further increased by methionine. Supplementary choline augmented the amount of arachidonic acid in phosphatidyl ethanolamine but not in phosphatidyl choline, whereas methionine increased this fatty acid in both phospholipids. Docosahexaenoic acid was relatively high in phosphatidyl ethanolamine when arachidonic acid was low. This increased amount of the $\omega 3$ polyenoic acid was not transferred to phosphatidyl choline.

DISCUSSION

Methionine was more effective than choline in increasing the amount of phosphatidyl choline, but, as found by others (9,10), not in reducing the total lipids in the liver. Since the direct route for the synthesis of phosphatidyl choline from CDP-choline and diglyceride involves fatty acids which more closely resemble those of the accumulated liver fat than those derived from phosphatidyl ethanolamine, Yamamoto (10) suggested that the direct route was more important in the removal of liver triglycerides.

It is well established that methyl groups are involved in the conversion of phosphatidyl ethanolamine to phosphatidyl choline (11-13,3). Although more methyl groups were supplied here by 1% choline than by 0.5% methionine, the latter appeared to be more effective in correcting the phospholipid alterations produced by the basal choline-deficient diet. In agreement with earlier work which indicated that the methyl groups of choline were not labile (9), Weingold (14) demonstrated their lack of utilization in the methylation of phosphatidyl ethanolamine. Also, the results of Haggard and Parks (15) suggested that

the incorporation of choline into phosphatidyl choline was independent of the methylation pathway.

The increased intake of food, and consequently of linoleate, in the group supplemented with methionine might make more of this fatty acid available for arachidonate synthesis, but there was no evidence that linoleate was a limiting factor.

In choline deficiency, there was an increased requirement for the synthesis of phosphatidyl choline from the sequential methylation of phosphatidyl ethanolamine. That was also the pathway for the synthesis of the species of phosphatidyl choline containing polyunsaturated fatty acids (16-19). The availability of methyl groups would thus be expected to affect the production of phosphatidyl choline. This appears to be a reasonable explanation for the amount and the fatty acid composition of phosphatidyl choline produced in the present investigation of choline deficiency and the remedial effect of supplementary methionine. The change in the composition of phosphatidyl ethanolamine is less readily explained. It was previously suggested that linoleic may be converted to arachidonic acid within the phosphatidyl ethanolamine molecule (7), but other phospholipids may be necessary for this synthesis. There is an indication, for example, that a desaturase requires the presence of phosphatidyl choline (20).

Since the phospholipid changes previously attributed to advanced choline deficiency were corrected by methionine, they should probably be considered to reflect a deficiency of methyl groups rather than of choline.

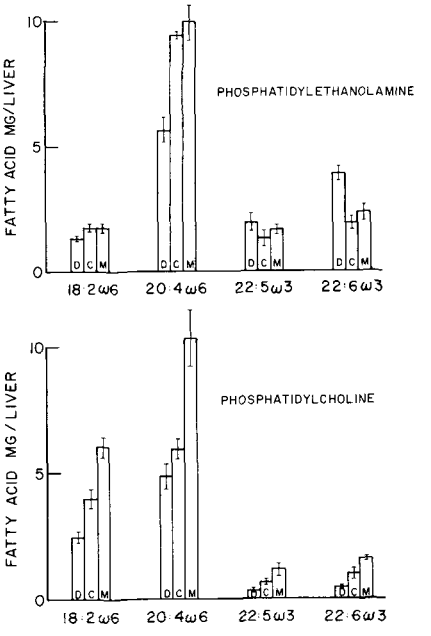

FIG. 3. Amounts of polyunsaturated fatty acids in liver phosphatidyl ethanolamine and phosphatidyl choline of the rats described under Figure 2.

ACKNOWLEDGMENTS

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Resistance to the Effect of Phospholipase A_2 of the Biliary Phospholipids During Incubation of Bile

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ABSTRACT

Mixtures of fresh bile of the rat and of isolated hepatic phospholipids (one or the other of these components having been labeled with ^3H oleic acid) were incubated either with heated rat pancreatic juice, at 37 C during periods of 1 and 3 hr, or with snake venom, at 25 C during periods of 17 and 36 hr, as sources of phospholipase A_2. After incubation, tritiated free oleic acid was measured since this acid was in the 2 position of both phospholipidic substrates. With heated pancreatic juice, no significant enzymatic hydrolysis of the bile phospholipids occured, but isolated hepatic phospholipids were readily attacked. With snake venom, the whole isolated hepatic phospholipids were very strongly hydrolyzed while biliary phospholipids were hydrolyzed to a much lesser extent.

INTRODUCTION

In previous experiments (1), small amounts of radioactive bile were obtained from a rat having received $9.10.^3H_2$ oleic acid by intravenous perfusion. About 70% of the bile radioactivity was present as phospholipids (PL). The bile was introduced into the duodenum of fat-fed rats bearing a lymphatic fistulae. The activity recovered in the lymph lipids during a collection time of 17 hr was only 10% of the radioactive bile lipids introduced. In short term experiments (1 hr) a large amount of ^3H oleic acid went into the portal blood; 50% of that radioactivity was present as PL. These results suggest that bile PL have been hydrolyzed at a slower rate than dietary PL and furthermore, that the two kinds of PL do not have the same way of absorption, since it is known that dietary PL are hydrolyzed in the intestinal lumen by the pancreatic juice phospholipase A_2 and that their fatty acids go into the intestinal lymph as triglycerides (2,3).

To prove that bile PL were protected against an enzymatic hydrolysis, we performed several in vitro experiments. Mixtures of fresh bile and isolated hepatic PL represented the substrate, one or the other of these components having previously been labeled with ^3H oleic acid. They were incubated either with heated pancreatic juice of rat or with snake venom as sources of phospholipase A_2 (E.C.3.1.1.4. phosphatide acylhydrolase). The distribution of radioactivity between lipid components was determined after the incubation.

MATERIALS AND METHODS

Collection of Bile

Bile was collected for 6 hr from rats weighing about 200 g; the cannula was near the liver, leaving the pancreatic duct intact (4). To obtain radioactive bile, the animals received, by intravenous perfusion, 17 mg of pure potassium ($9.10.^3H_2$) oleate 200 μ C (C.E.A., France) bound on 750 mg serum albumin dissolved in 4 ml physiological buffer during 40 mn.

Sources of Phospholipase A_2

Pure rat pancreatic juice was collected at 0 C after cannulation of pancreatic duct. Protein content of this juice was about 14 mg/ml. Before incubation, the pancreatic juice was heated at 70 C for 5 min.

Lyophilized snake venom (Naia-Naia) was provided by the Institut Pasteur (Paris, France).

Preparation of Lipids

Whole phospholipids or lecithins were prepared from lipid extracts of liver or bile by chromatography on silicic acid column (5). To obtain radioactive PL, an intravenous perfusion was performed as described above; bile was collected for a period of 6 hr; the liver was removed 2 hr later.

Triolein, dipalmitin and 1-monolaurin were commercial samples purified by thin layer chromatography (TLC).

Incubation Media

Each incubation medium, in experiments with pancreatic juice and with snake venom, contained 1 ml of bile (=3 mg of PL) and 7 mg of isolated PL (HPL) or lecithins (HL). The tritiated PL were present either as biliary PL (SA of fatty acid, 250,000 dpm) or as HPL (SA of fatty acids; 70,000 dpm).

The preparation process of the phospholipids was performed by introduction in each flask of the hepatic phospholipids (HPL or HL) dissolved in chloroform-methanol (1:1 v/v). The

TABLE I

Distribution of Radioactivity (%) in Lipid Fractions After Incubation[a] of a Mixture of Total Hepatic Phospholipids (HPL) and Fresh Bile at 37 C With Heated Pancreatic Juice[b]

Experiment No.	Composition of the mixtures	Radioactivity, %[c]					
		PL[d]	FFA	TG	DG+chol	MG	CE
1	^{3}H.18:1 bile + nonradioactive HPL + trypsin + Ca^{++}	65	5.5	6	8.5	15	0
		61	8	7	8.0	16	0
2	^{3}H.18:1 bile + nonradioactive HPL	70	5	4	10.0	11	0
		71	10	5	6.0	8	0
3	^{3}H.18:1 bile + nonradioactive HPL + mixture of nonradioactive glycerides + trypsin + Ca^{++}	67	4	7	10	12	0
		63	8	5	6	18	0
4	^{3}H.18:1 HPL + nonradioactive bile	78	5	3	3	11	0
		34	37	3	18	8	0
5	^{3}H.18:1 HPL + nonradioactive bile + trypsin + Ca^{++}	84	4	0	8	4	0
		67	16	0	10	7	0
6	^{3}H.18:1 HPL + nonradioactive bile + mixture of nonradioactive glycerides + trypsin + Ca^{++}	86	2	0	9	3	0
		66	20	0	8	6	0
7	Like 5	88	7	0	2	3	0
		72	24	0	2	2	0
8	Like 6	88	7	0	2	3	0
		77	18	0	3	2	0

[a]Incubation time was 1 hr except for experiments 7 and 8, 3 hr.

[b]The same sample of pancreatic juice was used for experiments 1, 3, 5 and 6, another for experiments 2 and 4, and yet another for experiments 7 and 8.

[c]Top figure is the control (no pancreatic juice), the bottom figure, the experiment (with pancreatic juice).

[d]Abbreviations: FFA, free fatty acids; TG, triglycerides; DG, diglycerides; chol, cholesterol; MG, monoglycerides; CE, cholesterol esters.

solvents were evaporated to dryness. One milliliter of bile was added and the flask was shaken at 30 C to obtain a homogenous mixture. In this way, the substrates were always identical and under the same physicochemical conditions.

Incubation mixtures with pancreatic juice were prepared according to the method of Magee et al.(6) and under constant shaking at 37 C for 1 or 3 hr; they contained, in addition to the PL substrates, 1 ml of pancreatic juice, borate buffer (pH 7,6) and up to 4,5 ml of total aqueous volume. Sometimes, trypsin (500 μg) and $CaCl_2$ (5 mg) were added (experiments 1,3,5,6,7 and 8) because the existence of an active form and of a zymogen of the phospholipase A_2 has been detected in the pancreatic juice. Trypsin converted this zymogen to its active form (7) and it was established that Ca^{++} is the activator of this enzyme (8).

Incubation mixtures with snake venom were prepared according to the method of Tattrie (9) and at 25 C during 17 and 36 hr periods. This medium had the same composition as the preceding one, but 100 μg of snake venom were used instead of 1 ml of pancreatic juice. We added 0.1 ml of a solution of $CaCl_2$, 0.005 M and 4 ml of ether.

In some experiments, 10 mg of triolein, 2 mg of dipalmitin and 3 mg of monolaurin were added to observe their effect on the hydrolysis rate. The controls had the same composition and were incubated as were the experiments, but they contained no enzyme. In both kinds of incubation, HPL were dissolved in bile just before the introduction of borate buffer and enzymes; 0.25 ml of N.HCl was added to stop enzymatic reaction.

Extraction and Separation of Lipids

Lipids were extracted with dimethoxymethane-methanol (4:1 v/v). The proteins were removed and solvents evaporated to dryness. Lipids were recovered in chloroform-methanol (1:1 v/v) and bile acids accompanied the lipids in this solvent mixture. Lipids were separated on 250 μ TLC plates, using 1/15 of the lipid material, developed in mixture, hexane-ether-acetic acid-water 90:20:2:3 v/v. In this system, conjugated bile acids had the same Rf as PL. [To check that biliary acids do not modify the separation of nonphosphorus lipids on TLC plates the following experiments were performed: 1 ml of bile mixed with 7 mg of radioactive free fatty acids (FFA) or triglycerides (TG) were incubated at 37 C during 1 hr. Lipids were extracted and separated as described in the text. All the radioactive material was recovered as FFA or TG.]

Spots were visualized using iodine vapor, scraped and introduced into a scintillation vial containing 1 ml of methanol; 2 hr later, 15 ml of scintillation liquid were added. Radioactivity was counted in a Packard Tri-Carb Scintillation Spectrometer, model 3324. Quenching was automatically corrected.

RESULTS

Position of ^3H Oleic Acid in the Hepatic and Bile PL

As the hydrolysis of radioactive substrates was followed by determination of the radioactivity of PL remaining in the media after the incubation, it was necessary to check the position of this acid in the labeled PL since it was established that phospholipase A^2 releases the fatty acid located on the 2 position of PL molecules.

The degradation of the HPL (Table II) with snake venom showed that 85% of tritiated oleic acid was located in the 2 position; after enzymatic hydrolysis of isolated bile lecithins (experiment not described here) the percentage of labeled oleic acid located in the 2 position was found in a similar proportion. This result agrees with other findings on human and sheep bile PL (10,11).

Rates of Hydrolysis of PL With Pancreatic Juice and Snake Venom

These rates can be calculated from the per cent of tritiated PL in the controls and in the experiments (Tables I and II).

The data indicate (a) that with heated pancreatic juice, no significant enzymatic hydrolysis of the bile PL occured, but that 11% to 50% of isolated HPL were hydrolyzed, and (b) that with snake venom, 65% to 85% of the isolated HL were attacked while only 17% to 27% of biliary PL were hydrolyzed.

Notice that the results were similar using three different samples of pancreatic juice (in experiments 1,3,5 and 6, as well as in experiments 2 and 4 and in 7 and 8) and two different samples of radioactive bile (one for experiments 2 and 9 and another for 1,3,10 and 11). The addition of trypsin or glycerides to the incubation media had no effect on the rate of hydrolysis.

Moreover, we should specify that it was normal to find some radioactivity in the different lipid fractions in bile collected on rats having received ^3H oleic acid by intravenous perfusion (the distribution of the radioactivity was about the same in both fresh bile and in controls); however, it was unexpected to find radioactivity in nonphosphorus-containing lipids in the controls (particularly in experiments 4 and 12, Tables I and II) containing

TABLE II

Distribution of Radioactivity (%) in Lipid Fractions After Incubation[a] of a Mixture of Hepatic Lecithines (HL) and Fresh Bile With Snake Venom

Experiment No.	Composition of mixtures	Radioactivity, %[b]					
		PL[c]	FFA	TG	DG+chol	MG	CE
9	^{3}H.18:1 bile + nonradioactive HL	70	2	2	10	16	0
		53	20	3	8	16	0
10	^{3}H.18:1 bile + nonradioactive HL	67	4	4	9	16	0
		49	33	2.5	7.5	10	0
11	^{3}H.18:1 bile + nonradioactive HL + nonradioactive mixture of glycerides	69	5	4	8	14	0
		42	37	5	6	10	0
12	^{3}H.18:1 HL + nonradioactive bile	65	15	7	10	3	0
		10	81	4	3	2	0
13	^{3}H.18:1 HL + nonradioactive bile	86	7	3	3	1	0
		11	80	4	4	1	0
14	^{3}H 18:1 HL + nonradioactive bile + nonradioactive mixture of glycerides	84	6	2	5	3	0
		10	83	2	4	1	0
15	Like 13	87	5	0	3	5	0
		23	70	0	6	1	0
16	Like 14	87	5	0	3	5	0
		24	69	0	5	2	0

[a]Incubation time was 17 hr except in experiments 15 and 16, 36 hr.

[b]Top figure is the control (no snake venom), the bottom figure, the experiment (with snake venom).

[c]Abbreviations: see Table I.

isolated HPL or HL, theoretically only substrates labeled in the media. This last fact is partially explained by the presence of small amounts of radioactive nonphosphorus lipids (2% to 7%) accompanying the HPL and running with the same fractions of nonradioactive bile during the analysis by TLC. Another explanation could be the spontaneous hydrolysis of fragile molecules as phosphatidic acids during the incubation.

DISCUSSION

These findings suggest that under our experimental conditions, bile PL are partially preserved against phospholipase A_2 hydrolysis and that isolated HPL in the presence of bile are hydrolyzed at a much lesser extent than HPL incubated without bile, since it is known that in this case hydrolysis is almost complete (7). These results explain our previous study (1).

Recent researchers (12,13) have indicated that bile had an inhibitory effect on some enzymes; our results show that this is partially true for rat pancreatic juice phospholipase A_2 acting on isolated HPL. Indeed, if no bile was present in the media, these PL would be hydrolyzed to a much higher extent. The snake venom enzyme was more active and less sensitive to the inhibitory effect of bile.

The differences observed in the rates of hydrolysis of bile PL and HPL were not due to differences in physicochemical conditions since both substrates were always mixed in the same proportions for each experiment. In addition, the conditions chosen in this study for the incubations were optimal for phospholipase activity (6,9). Particularly, an equal weight ratio was kept between the quantity of bile salts and of total PL in each medium. Since we have found, in agreement with other authors (13-15) that the bile contained 3 mg/ml of PL and 8 to 10 mg/ ml of bile salts for a collection time of 6 hr after surgical intervention, 7 mg of isolated PL were added in each medium.

The proportions of these components, PL and biliary salts, were thought good also by Olive and Dervichian (17) who studied the kinetics of hydrolysis of lecithins with snake venom according to the percentage of cholate in lecithin-cholate mixtures. They found a maximum rate of hydrolysis when the weight ratio of both substances was equal to 1.

In some cases glycerides were added to incubation media in order to make this comparable with the lipid composition of the intestinal lumen on fat-fed rats; no difference was found.

Our experiments do not explain the resistance of bile PL to the hydrolysis by pancreatic juice phospholipase A_2; these results suggest rather that in bile, PL must be engaged in some kind of complex (with proteins, biliary salts, sterols) which preserves them from enzymatic hydrolysis. In vivo experiments are in progress to answer this question.

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Fertility and Testicular Fatty Acid Composition in the Chicken as Influenced by Vitamin E and Ethoxyquin[1]

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ABSTRACT

The fatty acid composition of testicular lipids has been determined and related to fertility data from groups of dubbed White Leghorn cockerels after a 50-week feeding period on rations containing 10% safflower oil or coconut oil. Supplements of ethoxyquin or *d*-α-tocopherol acetate maintained fertility in birds raised on rations containing safflower oils. This response was associated with higher proportions of 22:4 ω6 and lower proportions of 18:2 ω6 in testicular lipids. Testes size was quite variable in the unsupplemented group with changes in fatty acid composition being more pronounced in the smaller testes. A multiple regression was calculated using data from those birds on the safflower oil ration. With a correlation ratio of 0.90 fertility was expressed as a function of testes size, semen concentration and the proportions of 18:2 ω6, 20:4 ω6 and 22:4 ω6 in testicular lipids. Despite the low intake of linoleate significant levels of polyunsaturated fatty acids were maintained in testicular lipids of birds fed the coconut oil rations. The major changes in fatty acid composition of testicular lipids produced by this variable was a decrease in the proportion of 18:2 ω6 and an increase in the proportion of 18:1.

INTRODUCTION

With the rat a vitamin E deficiency produces testicular degeneration and a characteristic change in the fatty acid composition of testicular lipids (1,2). The proportion of docosapentaenoic acid, 22:5 ω6 (This notation for fatty acids specified the number of carbons in the chain, the number of double bonds, and the number of carbon atoms after the terminal double bond in the molecule.), the major polyunsaturated fatty acid (PUFA), is markedly reduced while the proporation of arachidonic acid, 20:4 ω6, is increased. Witting has noted that this type of fatty acid response is characteristic of affected tissues of rats deficient in vitamin E (2).

On the other hand, testicular degeneration in cockerels resulting from a vitamin E deficiency is not as severe as with the rat (3) and the resulting sterility is reversible (4). This distinction may be related to the fact that in testicular lipids of the chicken a tetraenoic acid (22:4 ω6) rather than a pentaenoic acid is the major PUFA (5). To date the fatty acid composition of testicular lipids of chickens subjected to a vitamin E deficiency has not been studied.

In a recent study, Kuhns and Arscott (4) have evaluated the effectiveness of *d*-α-tocopherol and ethoxyquin in maintaining fertility in chickens fed rations containing 10% safflower oil as well as the effectiveness of these substances in restoring fertility in chickens subjected to an extended vitamin E deficiency. This paper reports fatty acid data from those birds used in the maintenance study, as well as a group of birds fed a ration containing 10% coconut oil. These results are interpreted with reference to fertility data.

EXPERIMENTAL PROCEDURES

Dubbed, White Leghorn cockerels were

[1]Paper No. 3050, Oregon Agricultural Experiment Station.

TABLE I

Dietary Variables

Diet	Dietary fat, 10%	Supplement, mg/kg		No. of birds
		Vitamin E[a]	Ethoxyquin[b]	
1	Safflower oil	32.4	—	6
2	Safflower oil	—	750	5
3	Safflower oil	—	—	5
4	Coconut oil	—	—	5

[a]Myvamix, 44 IU/g (*d*-α-tocopherol acetate) Distillation Products Industries.
[b]Santoquin (1,2-dihydro-6-ethoxy-2,2,4-trimethylquindine) Monsanto Chemical Co.

TABLE II

Influence of Dietary Variables on Fertility and Fatty Acid Composition of Testes

Fatty acid	Diet 1[a]	2[b]	3[c]	4[d]
16:0	20.0 ± 1.42[e]	19.4 ± 0.85	19.4 ± 0.85	18.9 ± 1.0
16:1	1.4 ± 0.11	1.3 ± 0.05	1.4 ± 0.19	1.5 ± 0.15
18:0	16.2 ± 0.77	16.0 ± 0.64	16.3 ± 0.71	15.7 ± 0.68
18:1	14.7 ± 2.79	16.2 ± 2.69	18.2 ± 3.86	20.2 ± 2.35
18:2 ω6	4.9 ± 0.77	4.6 ± 0.30	6.1 ± 2.55	2.6 ± 0.26
20:1	4.6 ± 0.39	4.2 ± 1.14	3.7 ± 1.49	4.6 ± 0.23
20:2	1.5 ± 0.15	1.2 ± 0.05	1.3 ± 0.22	1.5 ± 0.14
20:3 ω6	1.9 ± 0.41	1.7 ± 0.33	2.3 ± 0.58	1.8 ± 0.34
20:4 ω6	13.3 ± 0.63	13.5 ± 0.63	13.8 ± 2.58	12.3 ± 0.88
22:3 ω6	2.0 ± 0.41	2.0 ± 0.40	1.9 ± 1.19	2.7 ± 0.26
22:4 ω6	14.4 ± 1.50	15.6 ± 1.02	12.6 ± 1.76	12.3 ± 0.52
22:5	1.0 ± 0.28	1.0 ± 0.22	0.9 ± 0.37	1.2 ± 0.10
22:6 ω3	1.7 ± 0.74	1.2 ± 0.55	0.6 ± 0.44	1.9 ± 0.21
Testes weight g/kg body wt.	5.86 ± 1.87	7.44 ± 0.99	4.33 ± 2.68	5.74 ± 1.71
Fertility (%)[f]	69 ± 9	86 ± 11	11 ± 6	62 ± 17
Semen concentration (%)	11 ± 2	13 ± 2	5 ± 2	10 ± 2
Semen volume, ml	.65 ± .08	.55 ± .12	.51 ± .11	.47 ± .12

[a]Safflower oil and vitamin E.

[b]Safflower oil and ethoxyquin.

[c]Safflower oil.

[d]Coconut oil.

[e]Mean and standard deviation.

[f]Initial values: fertility 73%, semen concentration 14%, semen volume .71 ml.

placed on the semi-purified experimental rations at 33 weeks of age. Previously the birds had been fed conventional rations. Prior measurement of fertility, semen volume and body weight allowed for an initial equalizing of these factors in the four experimental groups. Specific details of experimental design and procedures have been reported previously (4).

Dietary variables significant to this study are given in Table I. In one instance safflower oil was replaced by an equal amount of coconut oil. Both the safflower oil and coconut oil were air-oxidized to reduce the level of endogenous tocopherols (6). A detailed description of the experimental rations is given elsewhere (6).

After 50 weeks on the experimental rations, the birds were killed, the testes excised and weighed, and the lipid extracted using chloroform-methanol. The fatty acid methyl esters were prepared using anhydrous HCl in methanol-ether (1:1) and analyzed by gas chromatography. Separations were obtained using a 6 ft x 1/8 in o.d. aluminum column of 15% ethylene glycol succinate on AW chromosorb P, 80-100 mesh at 190 C. A gas flow of 30 ml/min was used. Chain length and degree of unsaturation were established by hydrogenation and thin layer chromatography on silver nitrate-silicic acid. Details of the procedures used in our laboratory for fatty acid analysis have been given (7).

RESULTS AND DISCUSSION

The fatty acid composition of testicular lipids of cockerels observed in this study (Table II) compares quite closely to that reported by Nugara and Edwards (8) for birds 22 weeks of age; 20:4 ω6 and 22:4 ω6 were found to be the major PUFA. Compared with the fatty acid composition of testicular lipids from the rat (1), the chicken shows a higher proportion of stearate (16% vs. 6%) and a lower proportion of palmitate (20% vs. 35%). Crude lipid levels were found to be relatively constant among the four dietary groups at approximately 10 mg/g wet weight.

Observation of the birds fed the rations containing the safflower oil showed that the omission of ethoxyquin or vitamin E from the diet results in a decrease in the proportion of 22:4 ω6 in testicular lipids and a corresponding increase in the proportion of 18:1 and 18:2 ω6.

In the absence of added antioxidant the replacement of safflower oil with coconut oil did not change the level of 22:4 ω6 in testicular lipids. The level of 18:2 ω6 was

TABLE III

Variations in Fatty Acid Composition With Testes Weight in Birds From Diet 3 (Safflower Oil)

Fatty acid	Testes weight, g/kg body weight, %	
	1.07 (2)[a]	6.51 (3)[a]
16:0	20.1	19.0
16:1	1.2	1.5
18:0	15.7	16.7
18:1	18.1	18.2
18:2	9.2	4.0
20:1	2.1	4.8
20:2	1.2	1.5
20:3	2.9	1.9
20:4	15.2	12.9
22:3	0.6	2.8
22:4	11.5	13.3
22:5	0.8	1.1
22:6	0.8	0.4

[a]Number of birds in parentheses.

TABLE IV

Independent Variables Included in the Multiple Regression Equation

Variable	Partial regression coefficient	Student's t value (10 df)
Per cent 18:2	22.7	3.26
Per cent 20:4	-12.2	-2.59
Per cent 22:4	11.7	2.93
Semen concentration	5.81	3.20
Relative testicular size	6.69	1.85
Constant	-158	

reduced, however, with a concomittant increase in the level of 18:1 being observed.

After the 50-week feeding period, significant decreases in fertility and semen concentration were observed only in the males receiving Diet 3 (safflower oil, no antioxidant). The dietary variables did not produce significant changes in testes weight or semen volume. The requirement of dietary linoleate as a stressor confirms previous observations (6). Testicular lipids of birds receiving Diet 3 showed the highest level of linoleate.

In a previous note (9) reference was made to the presence of a polar component in the mixture of fatty acids esters the proportion of which seemed to increase in testes from birds not receiving antioxidant supplements. We have since established that this component was cholesterol (10). The suggestion that the proportion of cholesterol increases in testicular lipids of birds deficient in vitamin E would not be inconsistent with the observations of Bieri and Prival in vitamin E deficient rats (1). The cholesterol content was shown to remain constant whereas the content of phospholipid decreased.

The fatty acid data from those birds receiving Diet 3 (safflower oil, no antioxidant) are much more variable than those data from the three other dietary groups. This is due in part to a large variation in testes size (Table III). The lipid from the smaller testes showed lower levels of 22:4 ω6, 22:3 ω6 and 20:1 and higher levels of 18:2 ω6 and 20:4 ω6. A decrease in 22:4 ω6 coupled with an increase in 20:4 ω6 would parallel changes in fatty acid composition observed in affected tissues of vitamin E deficient rats (2). Even with this extended feeding period, the largest decrease in the level of 22:4 ω6 observed in this study was of the order of 20%. With the rat, the level of 22:5 ω6 would decrease by 70% in the absence of an antioxidant supplement. Despite the extended feeding period, three of the birds of Diet 3 maintained normal testes size and a fatty acid composition not too different from that observed in birds receiving antioxidant supplements.

Both Bieri and Prival (5) and Witting (2) have commented on the fatty acid composition of testicular lipids being particularly resistant to changes induced by dietary means. This situation apparently holds true for the chicken also since substitution of coconut oil which contains only 0.3% linoleate for safflower oil produced only small changes in the fatty acid composition of testicular lipids (Table II). One might expect the lower level of 18:2, but the levels of 20:4 ω6 and 22:4 ω6 which were maintained are quite surprising. The affinity of this tissue for the ω6 fatty acids is quite unique.

The fertility of the 16 birds raised on the rations containing safflower oil varied over a wide range and a stepwise multiple regression was calculated to obtain some indication of the relation between composition and function. Those variables which contributed significantly to the reduction of the residual sum of squares are listed in Table IV, together with the partial regression coefficients and Student t values which are indicative of the probability that the coefficients differ from zero. A correlation coefficient of 0.90 was obtained for the multiple regression. In simple regressions testicular size and semen concentration were correlated significantly only with the proportion of 18:2 ω6 ($r = -0.80$ and -0.73, respectively), and fertility with the proportion of 22:4 ω6 ($r = 0.63$). These data are suggestive rather than definitive, but they do reflect the complexity of the overall relation.

The assumption that a specific level of 22:4

ω6 is required in testicular lipids for fertility is not valid. Birds on Diet 3 (safflower oil, no antioxidant) and on Diet 4 (coconut oil) showed a comparable average level of 22:4 ω6; however, the average fertilities were 11% and 62%, and semen concentrations were 5% and 10%, respectively. Neither is atrophy of the testis an absolute requirement for sterility. With Diet 3 (safflower, no antioxidant) zero fertility was observed in two birds whose relative testes size was 0.80 and 6.91 g/kg body weight. The regression equation would implicate both of these variables; however, the relationship is obviously not a simple one. Bieri and Prival (1) also have experienced difficulty in attempting to correlate the morphological changes produced in the rat by a vitamin E deficiency with changes in the composition of testicular lipids.

This study is exploratory in nature and a clearer understanding of the relation between function and composition will require a more intensive study of the various aspects of sperm production and viability. The data are not inconsistent with an antioxidant role for vitamin E; however, the relatively high level of PUFA in testicular lipids of birds fed the coconut oil poses an interesting question. Fertility was maintained in this case without an antioxidant supplement. It would seem that the vitamin E is required not so much to maintain some required level of PUFA as to prevent autoxidation and consequent damage. Safflower oil presumably supplies a surplus of PUFA, thus predisposing the tissue to oxidative damage.

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Lipids of Bovine Thyroid

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ABSTRACT

The lipid composition of the freshly slaughtered bovine thyroid tissue has been investigated. The phospholipid patterns of microsomal and mitochondrial fractions obtained from homogenates of bovine thyroids have also been determined. They resemble the phospholipid composition of the corresponding subcellular fractions from other tissues. The fatty acid composition of the various phospholipid species of these subcellular components have also been estimated by gas liquid chromatography. These analyses reveal that the fatty acids are not particularly characteristic of the subcellular organelle but tend to be characteristic of the lipid species. There is a high percentage of nervonic acid (C24:1) in all the subcellular phospholipid species examined.

Phospholipid patterns of sheep and dog thyroids have been studied (1,2) and phospholipids have been postulated as the possible mediators of thyroidal iodide transport (3,4). The unsaturated fatty acids of phospholipids are considered to be the probable site of iodination (4). For example, nervonic acid (24:1) present at the β-position of the human thyroidal phosphatidyl choline has been implicated in the role of binding and carriage of iodide ion (5). The enhanced ^{32}P incorporation into phospholipids of thyrotropin-stimulated thyroids has been well documented (2,5,7) and the mitochondria and microsomes of these stimulated thyroids are the subcellular organelles which exhibit increased turnover of phospholipids (8,9). However, there are no reports on the subcellular phospholipid distribution in the thyroid tissue. The present studies were undertaken to provide information on the proportions of individual phospholipids of the bovine thyroidal mitochondrial and microsomal fractions. The fatty acid composition of the individual phospholipid species of these organelles have been determined by gas liquid chromatography (GLC).

MATERIALS AND METHODS

All solvents employed for extraction and chromatography were Analar grade and were redistilled before use. Kieselgel H was obtained from E. Merck, Darmstadt, Germany. Standard GLC pure fatty acid methyl esters were from Applied Science Lab., State College, Pa. and were donated by Fred Snyder, Oak Ridge Laboratories.

Bovine thyroids from freshly slaughtered animals were transported to the laboratory frozen in ice. After removal of adhering fatty material and connective tissue, the tissue was sliced, washed in saline, blotted with filter paper, weighed and taken for lipid extraction. Each analysis contained thyroid slices pooled from at least five different thyroids.

The thyroid slices were homogenized in $CHCl_3$/MeOH (2:1 v/v) and the total lipids were prepared by the procedure of Folch et al. (10).

Thin Layer Chromatography

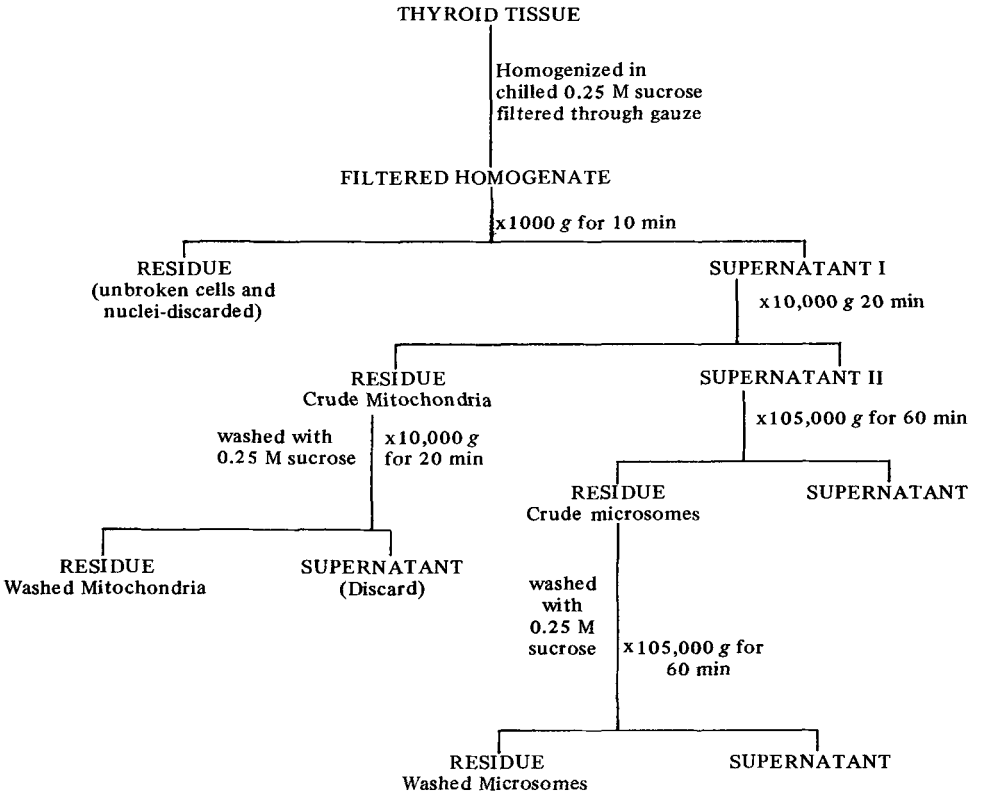
All thin layer separations were carried out in glass tanks containing chromatography paper as wicks to facilitate solvent equilibration. Thin layers of Kieselgel H (1 hr activation) were used to separate the phospholipid classes using the solvent system chloroform-methanol-acetic acid-water (50:25:8:4 v/v). Lipids resolved by thin layer chromatography (TLC) were sprayed with concentrated H_2SO_4 and charred. Phospholipids were also visualized by exposure of the TLC plates to iodine vapor. Phospholipid zones were scraped and quantitatively transferred to test tubes, and the lipids were extracted from the silica gel for phosphorus estimation employing three batches of the same solvent system used for the development of the chromatograms. Total lipid P and individual phospholipid P were determined by Bartlett's method (11). Cholesterol and cholesterol esters were estimated by the method of Abell et al. (12).

Gas Liquid Chromatography

Methyl esters of the fatty acids of the individual phospholipids were prepared by refluxing the Kieselgel scrapings of phospholipid zones for 5 hr with H_2SO_4 (1%) in dry methanol.

Panchromatograph (Model 12103, W.G. Pye and Co., U.K.) equipped with a ^{90}Sr ionizing detector was used in this study.

A 152 cm x 30 mm o.d. pyrex glass column



Scheme 1. Procedure for subcellular fractionation of bovine thyroidal homogenate.

packed with 15% EGS on 80-100 mesh chromosorb W was used for all analysis. Temperature of the column was 178-184 C; temperature of the detector, 225 C; argon flow, 60 ml/min.

Fatty acids were identified by: (a) comparison of relative retention times with standard compounds and with those given in literature, (b) analysis of GLC results before and after hydrogenation to fix the identity of unsaturated fatty acids, and (c) by plotting retention times relative to methyl stearate against chain length and determining the chain length of unknown peaks from the curve.

Quantitation of fatty acids was done by the triangulation method and results were expressed as per cent of the total fatty acids obtained by GLC.

Sub-Cellular Fractionation

The thyroid slices were homogenized with four volumes of chilled 0.25 ml sucrose solution and subcellular fractions were prepared by the differential centrifugation of the homogenate. The entire procedure for separation of the subcellular organelles was carried out at 4 C. The subcellular fractionation procedures employed were essentially the same as those of Kogl and van Deenen (8) and that of DeGroot and Carvalho (15). They are schematically shown in Scheme 1.

RESULTS

The lipid composition of the bovine thyroids is the following. The total lipids, five samples, form 1.8 ± 0.47/100 g of the wet weight of the fresh tissue (the value preceded by ± is the standard deviation); lipid phosphorus, four samples, forms 19.1 ± 4.1 μg/mg lipid; total cholesterol, five samples, forms 120.0 ± 15.0 μg/mg lipid; free cholesterol, five samples, forms 102.0 ± 9.6 μg/mg lipid; cholesterol ester, five samples, forms 18.0 ± 4.4 μg/mg lipid (cholesterol ester values were obtained by subtracting free cholesterol from total cholesterol).

The distribution of phospholipids in the whole thyroid tissue, and the thyroidal, mitochondrial and microsomal fractions are given in Table I. Phosphatidyl choline (PC) (42%) is the predominant phospholipid followed by phosphatidyl ethanolamine (PE) (26%), sphingomy-

TABLE I

Phospholipid Patterns in Bovine Thyroid and Its Subcellular Distribution[a]

Phospholipid species	Whole thyroid, %	Mitochondrial fraction, %	Microsomal fraction, %
Lysolecithin	3.0 (1.5-4.0)	3.5 (3.0-4.0)	2.0 (1.5-3.0)
Sphingomyelin	18.0 (15.5-19.5)	14.0 (12.0-16.0)	16.0 (15.0-18.0)
Phosphatidyl choline	42.0 (41.0-44.0)	36.0 (34.0-39.0)	46.0 (45.0-47.0)
Phosphatidyl serine + Phosphatidyl inositol	11.0 (10.5-11.6)	12.0 (10.5-14.0)	12.0 (11.0-14.0)
Phosphatidyl ethanolamine	26.0 (24.0-28.5)	34.5 (34.0-35.0)	28.0 (26.0-31.0)

[a]The results are the percentage of individual phospholipid P as determined from TLC. Each value represents the mean of three triplicates. The value given in parentheses are the ranges. Cardiolipin and phosphatidic acid which move with the solvent front were not estimated.

elin (18%), phosphatidyl serine (PS), and phosphatidyl inositol (PI) (11%) and lysolecithin (3%) in the whole thyroid tissue lipids. Phospholipids constitute the major proportion of the lipids of both subcellular fractions, mitochondria and microsomes, analyzed. Total lipid P in the mitochondria and microsomes were 28.5 μg P/mg lipid and 31.5 μg/mg lipid, respectively. More PE appears to be in mitochondria (34.5%) than in microsomes (28.0%); the predominant phospholipid of the microsomes is PC (46%). In mitochondria, PC (36%) and PE (34.5%) together constitute more than 70% of the phospholipids. Cardiolipin, shown to be localized in mitochondria of all tissues examined, has not been measured in our studies as they travel along the solvent front with phosphatidic acid (PA) and other lipids.

The fatty acid profile of the individual phospholipids of mitochondrial and microsomal fractions analyzed exhibit variation among themselves (Tables II and III). The same phospholipid species, whether localized in the mitochondrial or microsomal fractions, show similarity in their fatty acid content. However, individual phospholipids, when compared within each fraction, show striking variation in their proportions of fatty acids. Thus, PC in both fractions show the highest percentage of palmitic acid (37.0% and 40.0%) followed by oleic

TABLE II

Fatty Acid Composition of Individual Phospholipids of Bovine Thyroidal Mitochondrial Fractions[a]

Fatty acid	Phosphatidyl choline	Phosphatidyl ethanolamine	Sphingomyelin	Phosphatidyl inositol + phosphatidyl serine
$C_{12:0}$	1.77	9.85	5.00	9.63
$C_{14:0}$	3.03	11.13	4.37	11.65
$C_{14:1}$	1.91	16.16	6.39	4.35
$C_{16:0}$	40.02	17.50	17.75	27.83
$C_{18:0}$	16.85	13.38	8.59	9.07
$C_{18:1}$	17.44	15.15	5.91	10.46
$C_{18:2}$	2.90	3.27	2.57	0.69
$C_{18:3}$	—	—	3.01	14.00
$C_{20:0}$	—	—	—	—
$C_{20:?}$	7.93	3.16	3.83	1.12
$C_{22:0}$	0.76	1.46	15.68	8.69
$C_{22:1}$	4.11	3.56	3.46	1.70
$C_{22:?}$	—	2.07	15.92	12.21
$C_{24:0}$	0.13	0.77	1.65	—
$C_{24:1}$	3.97	2.92	19.90	—
$C_{22:5}$	—	3.94	—	—
$C_{22:6}$	—	2.51	1.89	—

[a]Results are expressed as area per cent of total fatty acids as determined by GLC. Results given are the arithmetic mean of three individual samples. Experimental details in text.

TABLE III

Fatty Acid Composition of Individual Phospholipids of Bovine Thyroidal Microsomal Fractions[a]

Fatty acid	Phosphatidyl choline	Phosphatidyl ethanolamine	Phosphatidyl inositol + phosphatidyl serine	Sphingomyelin
$C_{12:0}$	1.12	5.75	9.14	5.32
$C_{14:0}$	1.63	6.58	7.95	8.42
$C_{14:1}$	1.33	16.15	4.08	19.39
$C_{16:0}$	37.55	17.17	27.19	20.33
$C_{18:0}$	15.22	12.15	12.10	6.96
$C_{18:1}$	24.36	22.42	10.22	5.86
$C_{18:2}$	6.72	3.68	1.70	1.93
$C_{18:3}$	0.30	1.52	3.02	1.63
$C_{20:0}$	0.66	1.78	0.67	—
$C_{20:?}$	3.80	2.70	1.16	1.19
$C_{22:0}$	3.76	4.10	5.50	12.20
$C_{20:4}$	—	9.39	10.16	—
$C_{22:?}$	1.18	5.10	10.63	9.08
$C_{24:0}$	—	—	—	3.46
$C_{24:1}$	4.90	4.56	4.03	6.00
$C_{22:5}$	—	1.07	—	—
$C_{22:6}$	—	1.20	—	—

[a]Results are expressed as area per cent of total fatty acids as determined by GLC. Results are arithmetic mean of three individual samples. Experimental details given in text.

acid (24.0% and 27.0%). The other phosphatides, PE, sphingomyelin, PS and PI contain a very high percentage (about 20% to 30%) of fatty acids with chain lengths shorter than 16:0. All the phosphatide species contain a significant proportion of nervonic acid (24:1). Mitochondrial sphingomyelin contains as much as 19% of this fatty acid. Considerable amounts of 22:0 is also present in this phospholipid.

DISCUSSION

The only available reports on phospholipid distribution in thyroids are those of Freinkel (1) and Scott et al. (2). Freinkel has shown in sheep thyroids that the alkali stable phosphorus comprised 20% of the total lipid phosphorus. The present data obtained by the direct determination of phosphorus of bovine thyroidal phospholipids from the Kieselgel plates are in good agreement. Scott et al. (2) have estimated the amounts of plasmalogen, alkyl ether lipids and PA, apart from the other major phosphatides in two dog thyroids. The methods employed in the present study do not resolve these phosphatides.

The general thyroidal lipid classes of both the whole tissue and the subcellular fractions show no striking differences when compared with the lipid classes of various other tissues. The distribution of phospholipids of the thyroidal mitochondrial and the microsomal fractions show quite a distinctive pattern, however, their fatty acid profiles do not reveal any significant organelle specificity. In mitochondria, PE and PC are present in equal amounts; in microsomes, PC is almost 1.5 times that of PE. Similar phospholipid patterns in subcellular organelles of various tissues have been reported (13).

The fatty acid patterns in the individual phospholipids of mitochondria and microsomes show a lack of differential subcellular distribution. That they are not particularly characteristic of the organelle but they tend to be characteristic of the lipid species has been demonstrated by many workers. Villki and Jaakonmaki (5) have reported a significant percentage of nervonic acid (24:1) in human PC at the β-position and have demonstrated its importance in complexing iodide. They have postulated an iodide ion carrier role for this species of phospholipid. The fatty acid analysis of thyroidal neutral lipids and phospholipids or the fatty acid patterns of the subcellular organelles studied do not show the presence of this fatty acid species perhaps due to its dilution to low concentrations when these components are analyzed. However, the individual phospholipid fatty acids of the mitochondria and microsomes showed a significant percentage of this component. Mitochondrial sphingomyelins showed a particularly high (19%) content of this fatty acid. If the presence of this fatty acid in the phospholipid molecule is a prerequisite for iodide binding, then all the phospholipid species might be expected to complex iodide and sphingomyelin should exhibit this iodide-

complexing phenomenon in higher proportions. However, sphingomyelin is almost inert in this regard (4,14). The results of Nagashima and Suzuki (4) and those of Shah and Shownkeen (14) from this laboratory show the presence of an unknown fraction with the highest iodide-complexing capacity. GLC analysis of this unknown fraction from sheep thyroids contain no traceable quantities of nervonic acid (unpublished data).

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The Triglyceride Composition of *Moringa concanensis* Seed Fat

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ABSTRACT

Moringa concanensis seed fat and its randomized product have been subjected to pancreatic hydrolysis. Glyceride compositions have been calculated from the original fatty acid composition and those of the monoglycerides produced by hydrolysis. The per cent GS_3 content of the interesterified product has also been determined by the combined techniques of thin layer chromatography on silver nitrate impregnated silica gel and colorimetry.

INTRODUCTION

Moringacea is a small family composed of the species, *Moringa olifera* (synonym, *M. pterygosperma*), *M. concanensis* and *M. aptera*, all native to India. *M. concanensis* is a rapidly growing tree, even in poor soil, and its green seed pods and flowers are used for food in India. Moringa oil (yield, 30-49% of the kernels) seems to be a promising one for exploitation in pharmaceutical and other allied industries because of its reportedly unusual resistance to the development of rancidity (1,2).

Several investigators (3-9) determined the fatty acid composition of *M. olifera* seed fat. Most of these studies were based on lead salt separation of the mixed fatty acids. Recently, Sengupta and Gupta (10) determined the fatty acid composition of the seed fats of *M. olifera* and *M. concanensis* by combination of the techniques of urea adduct segregation, UV spectrophotometry and quantitative paper chromatography. The fatty acid composition of this oil, as reported by these investigators, is interesting as it is composed of 25-30% saturated acid and 75-70% oleic acid. The triglyceride composition of the seed fat of *M. olifera* has been examined earlier (7,11) by the oxidation method of Kartha (12). This paper describes the determination of the triglyceride composition of *M. concanensis* seed fat by combination of enzymatic hydrolysis, thin layer chromatography (TLC) and gas liquid chromatography (GLC) techniques. The investigation was also extended to randomized oil to study the changes in the triglyceride composition effected by interesterification.

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EXPERIMENTAL PROCEDURES

Materials and Methods

M. concanensis seed kernels were procured from the market of Varanasi in Northern India. Chromatographic standards of tripalmitin, dipalmitin, monopalmitin, fatty acids and the methyl esters were obtained from The Hormel Institute, Sigma Chemical Co. and Calbiochem Inc., U.S.A.

Lipolysis was carried out as suggested by Coleman (13) at pH 8.5 and 37.5 C using a purified pork pancreatic lipase preparation with the addition of Ca^{++} ions and bile salts. The partial glycerides were separated on a thin layer

TABLE I

Characteristics of the Seed Fat and the Mixed Fatty Acids (MFA) of *M. concanensis*

Characteristics	Oil	MFA
Per cent free fatty acids (as oleic)	3.4	
Saponification equivalent	301.2	282.4
Iodine value, Wij's 30 min	67.2	71.2
Unsaponifiable, %	1.7	
$E^{1\%}_{1\ cm}$ at 268 mμ isomerized at 170 C for 15 min	—	Negligible
$E^{1\%}_{1\ cm}$ at 234 mμ isomerized at 180 C for 60 min	—	16.2
$E^{1\%}_{1\ cm}$ at 234 mμ unisomerized	—	Negligible
Per cent linoleic acid	—	1.8
Per cent monoethenoid acid	—	75.5
Per cent saturated acid (by difference)	—	22.7

TABLE II

Fatty Acid Composition of *M. concanensis* Seed Fat, mole %

Fatty acids	Mole %
$C_{14:0}$	0.1
$C_{16:0}$	15.6
$C_{17:0}$	0.2
$C_{18:0}$	3.0
$C_{20:0}$	2.7
$C_{22:0}$	4.3
$C_{16:1}$	3.9
$C_{17:1}$	0.1
$C_{18:1}$	66.0
$C_{20:1}$	2.4
$C_{18:2}$	1.7

(0.3-0.4 mm) of silica by developing with a solvent system of *n*-hexane, diethyl ether and acetic acid (85:15:1). The monoglyceride fraction, detected with 2'7'-dichlorofluorescein, was extracted with hot alcohol. The monoglyceride and the original triglyceride samples were then saponified, the free acids liberated, extracted and converted to methyl ester by acid catalyzed esterification process. GLC was carried out with an F and M analytical gas chromatograph (Model 700-R12) equipped with flame ionization detector. The column (6 ft x 1/4 in.) packed with 10% polyester of diethyl glycol adipate on 60-80 mesh gas chrom-Z was operated at 180 C with a carrier gas flow of 40 ml/min. Peak areas were determined as the product of peak height and the width at half height; the weight percentages obtained were converted to mole percentages.

The interesterified *M. concanensis* seed fat was fractionated on a silver nitrate-impregnated silica gel layer using the technique proposed by Barrett et al. (14) and the amount of GS_3 thus separated was determined quantitatively by the method of Litchfield et al. (15) based on the estimation of glycerol using the chromotropic acid color reaction.

The seed fat of *M. concanensis* was randomized at 30 C by the process suggested by Chakrabarty and Bhattacharyya (16). A solution of 100 g refined and bleached oil in 150 ml

TABLE III

Fatty Acid Composition (Mole %) of the 2-Monoglycerides

Fatty acids	Moringa oil	Randomized Moringa oil[a]
$C_{16:0}$	4.9	34.2
$C_{18:0}$	0.5	6.9
$C_{16:1}$	1.8	—
$C_{18:1}$	92.8	58.9

[a]Slip point 19 C; randomized at 30 C for 60 min.

TABLE IV

Triglyceride Composition[a] (mole %) of *M. concanensis* Seed Fat and Its Interesterified Product

Glycerides	Seed fat	Randomized product
SSS		
PPP	0.2	0.2
PPSt	0.3	0.5
StPSt	0.1	0.5
PStP	—	—
StStP	0.1	0.1
StStSt	—	0.1
Total	0.7	1.4
SSU		
PPO	1.1	3.2
PPU	0.2	0.6
StPO	0.8	5.4
StPU	0.2	1.0
PStO	0.1	0.7
StStO	0.1	1.1
StStU	—	0.2
PStU	—	0.1
Total	2.5	12.3
SUS		
POP	4.3	0.3
POSt	5.9	0.9
StOSt	2.0	0.8
PUP	0.1	—
PUSt	0.1	—
StUSt	—	—
Total	12.4	2.0
USU		
OPO	1.4	16.5
OPU	0.6	5.8
UPU	0.1	0.5
OStO	0.1	3.3
OStU	0.1	1.2
UStU	—	0.1
Total	2.3	27.4
SUU		
POO	20.9	5.5
POU	4.4	1.0
StOO	14.4	9.4
StOU	3.1	1.7
PUO	0.4	—
PUU	0.1	—
StUO	0.3	—
StUU	0.1	—
Total	43.7	17.6
UUU		
OOO	25.6	28.4
OOU	10.9	10.0
UOU	1.2	0.9
OUO	0.5	—
OUU	0.2	—
UUU	—	—
Total	38.4	39.3

[a]The fatty acids have been grouped as, 'P-$C_{14:0}$, $C_{16:0}$ and $C_{17:0}$'; 'St-$C_{18:0}$, $C_{20:0}$ and $C_{22:0}$; 'O-$C_{18:1}$' and 'U-$C_{16:1}$, $C_{17:1}$, $C_{20:1}$ and $C_{18:2}$.

TABLE V

Fatty Acid Composition of the Seed Fats of Moringaceae Family

Species	Per cent weight									
	Saturated acids						Unsaturated acids			
	C_{14}	C_{16}	C_{18}	C_{20}	C_{22}	C_{24}	$C_{18:2}$	$C_{18:1}$	$C_{16:1}$	$C_{20:1}$
M. pterygosperma										
(5)	1.5	3.6	10.8	—	6.3	0.1	3.8	68.9	—	—
(9)	1.6	3.9	11.5	—	6.6	0.2	—	72.2	—	—
(6)	—	5.5	7.8	2.7	1.2	5.3	0.8	75.8	0.9	—
(7)	—	9.3	7.4	8.1	—	—	—	65.7	—	—
(10)	—	3.1	8.0	7.8	3.5	5.8	0.8	71.0	—	—
M. concanensis										
(9)	—	5.5	7.8	2.7	1.2	5.3	0.8	75.8	0.9	—
(10)	—	11.7	3.8	2.4	4.1	0.6	0.9	75.5	—	—
(Present work[a])	0.1	14.3	3.0	3.0	5.2	—	1.7	66.3	3.5	2.6

[a]It also indicates the presence of $C_{17:0}$, 0.2% and $C_{17:1}$, 0.1%.

n-hexane was taken in a three-necked flask and to it was added, drop by drop, the catalyst sodium methoxide (methanolic solution, 0.4% w/w of the oil solution). Rearrangement was continued to the equilibrium state. After 60 min the randomized sample was isolated by the usual procedure after destroying the catalyst with dilute hydrochloric acid.

RESULTS

On analysis by standard procedure, the seed fat of *M. concanensis* and the mixed fatty acids obtained from it showed the characteristics given in Table I. Methyl esters of the mixed fatty acids were next analyzed by GLC. The results are shown in Table II.

The fatty acid compositions of the 2-monoglycerides obtained from lipolysis of the Moringa oil and its interesterified product as determined by GLC are given in Table III.

The triglyceride compositions of the *M. concanensis* seed fat and its randomized product were next calculated from the fatty acid composition of the original triglyceride and the 2-monoglycerides formed, using the assumption of Vanderwal (17) and Coleman (18). While calculating, the fatty acids have been grouped (19) as '$P\text{-}C_{14:0}$, $C_{16:0}$ and $C_{17:0}$'; '$St\text{-}C_{18:0}$, $C_{20:0}$ and $C_{22:0}$'; '$O\text{-}C_{18:1}$' and '$U\text{-}C_{16:1}$, $C_{17:1}$, $C_{20:1}$ and $C_{18:2}$'. The results are given in Table IV.

The percentage GS_3 content of the randomized *M. concanensis* seed fat as determined by the method developed by Litchfield et al. (15) was found to be 1.5.

DISCUSSION

The results of the present investigation along with the findings of the earlier workers on the fatty acid compositions of the seed fats of the Moringaceae family are shown in Table V.

The fatty acid composition of *M. concanensis* seed fat as determined by GLC thus agrees well with that determined by Sengupta and Gupta (10) utilizing the combined techniques of urea adduction and quantitative paper chromatography. Though the triglyceride composition of *M. concanensis* has not been investigated earlier, Rao et al. (7) and Kartha and Upadhyay (11) have studied earlier the triglyc-

TABLE VI

Triglyceride Composition (mole %) of Moringacea Seed Fat

Glycerides	*M. pterygosperma* (7)	*M. pterygosperma* (11)		*M. concanensis* (present work)
		North India	South India	
GS_3	1.4	—	—	0.7
GS_2U	23.4	16.0	18.3	14.9
GSU_2	25.6	37.3	25.2	46.0
GU_3	49.6	46.7	56.5	38.4

eride composition of *M. pterygosperma* seed fat of similar fatty acid composition utilizing the oxidation method. These data, along with the findings of the present investigation, are shown in Table VI.

Table III shows the preponderance of C_{18} unsaturated acids in the 2-positions of the triglycerides of *M. concanensis* seed fat. According to the theory of Gunstone (20) the 2-position of the triglycerides is preferentially esterified with C_{18} unsaturated acids as far as the composition allows and thereafter the remaining acids are distributed randomly among the unoccupied positions. The present results agree fairly closely with this theory but not completely, since they indicate about 5% of the saturated acids in the 2-position. The present findings agree more with the observation of Coleman (21), who reported 4.4% saturated acids in the 2-positions of the triglyceride of Karanja oil, containing 26.6% of saturated acid. The proportions of the six possible glycerides as calculated by the proposed formulae of Coleman (21) agree well with the findings of the present investigation (Table IV), the two sets of data being 'SSS-0.4, 0.7'; 'SSU-1.2, 2.5'; 'SUS-13.7, 12.4'; 'USU-1.0, 2.3'; 'SUU-45.7, 43.7' and 'UUU-38.0, 38.4' per cent, respectively. Table III further shows that during the process of interesterification the proportions of saturated acids in the 2-position increased from 5.4% in the seed fat to 41.1% in the randomized product. The triglyceride compositions of these products (Table IV) as calculated on the basis of the 1,3-random, 2-random distribution hypothesis also gives an idea about the changes in the proportions of the six possible glycerides effected by the process of interesterification. The content of GS_3 and GU_3 changed from 0.7% to 1.4% and from 38.4% to 39.3%, respectively, by this process of randomization. The proportion of GS_3 (1.4%) content of the randomized product as calculated from the lipolysis data is also in close agreement with that (1.5%) determined by the method developed by Litchfield et al. (15).

The high content of oleic acid (66.3%) and the low content of linoleic acid (1.7%) make the fatty acid composition of *M. concanensis* seed fat interesting for utilization in the pharmaceutical industry as a formulation ingredient. The 25.7% of triolein also indicates a possibility of exploitation of this seed. Further studies are being continued to develop a process for the preparation of oleic acid and triolein from the seed fat of *M. concanensis*.

ACKNOWLEDGMENT

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Fluorescent Lipids of Bovine Brain White Matter

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ABSTRACT

Two fluorescent lipids were isolated from a chloroform-methanol (2:1 v/v) extract of bovine brain white matter by two-step preparative thin layer chromatography on Silica Gel G. The first step was performed with chloroform-diethyl ether-acetic acid (70:30:1 v/v) as developing solvent and revealed a single fluorescent band below the solvent front. The band was scraped off, and the lipid was eluted in chloroform and reapplied to Silica Gel G plates. In the second step, benzene-methanol-ethyl acetate (85:10:5 v/v) as developing solvent revealed two fluorescent bands (A and B) with R_f values of 0.72 and 0.65. The lipids were eluted and the UV and visible absorption spectra were measured in heptane, as were the excitation and fluorescence spectra. Sulfuric acid absorption spectra (2 and 24 hr treatment at 22 C) as well as the resulting excitation and fluorescence spectra were also determined. The fluorescent lipids reacted positively in a number of nonspecific color tests for steroids, but the chemical nature of these minor components of the neutral lipid fraction remains to be established.

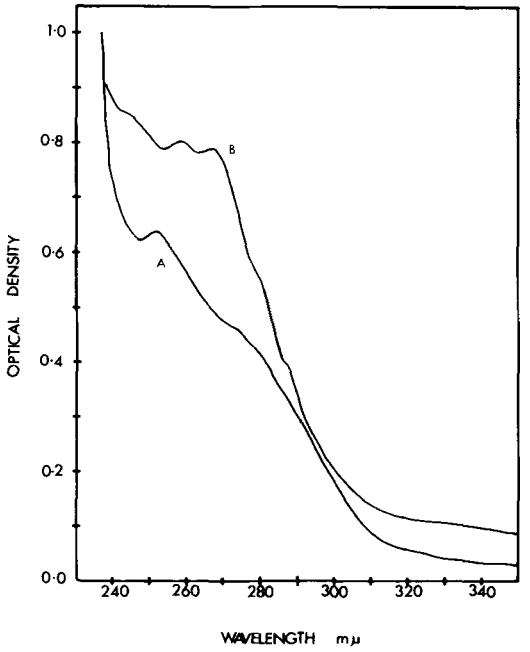

FIG. 1. Absorption spectra of fluorescent lipids A and B in heptane. Absorption maxima: A, 252 nm; B, 256 and 268 nm. These measurements and in Figure 4 were made in a Cary recording spectrophotometer.

INTRODUCTION

During studies on cholesterol, small amounts of two fluorescent lipids were found in the neutral lipid fraction of bovine white matter, chromatographed on thin layers of Silica Gel G. The lipid spots which fluoresced under UV light became rose-colored in daylight after spraying and heating with sulfuric acid, but were clearly distinct from cholesterol and its esters. Minor components of a lipid fraction are difficult to detect and isolate. However, after the bulk of the lipid components have been removed by preparative thin layer chromatography (TLC), the concentrate of minor components can be resolved further in a second preparative step (1). This paper describes the use of this strategy to prepare the fluorescent lipids and tests performed to try to characterize them. A preliminary report has been published (2).

MATERIALS AND METHODS

Extraction Procedures

Bovine (steer) brains from freshly slaughtered animals were brought to the laboratory in an ice bucket (transportation time 1 hr). The white matter was separated from the whole brain and the lipids were extracted from the fresh tissue or from stored frozen tissue. For each gram wet weight of white matter, 19 volumes of chloroform-methanol (2:1 v/v) were added and an extract was prepared in a glass homogenizer with teflon pestle. The chloroform-methanol insoluble residue was removed and the lipid extract was washed once according to Folch et al. (3). The lower phase was concentrated under N_2 at 37 C. The precipitated proteolipid protein and a small amount of water were removed by centrifuging in a clinical centrifuge. The chloroform phase was recovered. A small volume of chloroform was used to wash the protein precipitate and the chloroform soluble material was combined with the previously collected chloroform phase con-

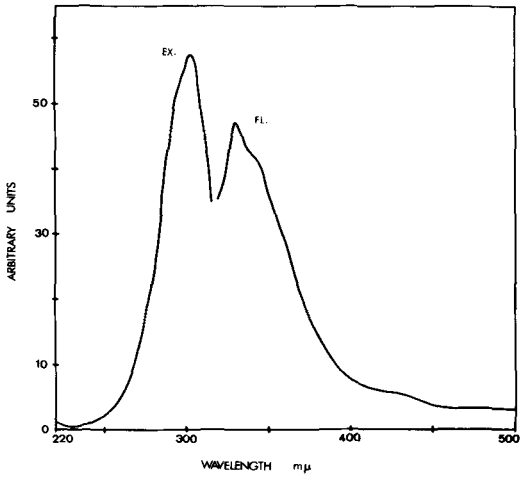

FIG. 2. Excitation and fluorescence spectra of lipid A in heptane. Excitation maximum, 300 nm; fluorescence maximum, 330 nm. These measurements and in Figures 3, 5 and 6 were made in spectrofluorometer Model SF-1 (Baird Atomic, Inc.), and are uncorrected.

taining the lipids.

Preparative TLC

Preparative TLC was carried out in two steps. The first solvent system (chloroform-diethyl ether-acetic acid, 70:30:1 v/v) was used to separate the neutral lipid fraction from the other brain lipids. The second solvent system (benzene-methanol-ethyl acetate, 85:10:5 v/v) was used to separate the two fluorescent lipids from one another. The TLC plates (1 mm in layer thickness) were activated at 110 C for 4 hr and stored at 37 C in an oven prior to use. To exclude any possible UV absorbing or fluorescent contamination from the silica gel, the coated plates were prewashed in the respective developing solvent and left in the oven at 37 C for several hours prior to use. The concentrated lipids in chloroform were applied in an even streak parallel to the lower edge of the plate. In the first step, the plate was developed twice in chloroform-diethyl ether-acetic acid (70:30:1 v/v). Between the two runs, the solvent was allowed to evaporate in a hood. Double development produced further relative migration of the faster moving components of the neutral lipid fraction, such as would have been obtained with a longer plate.

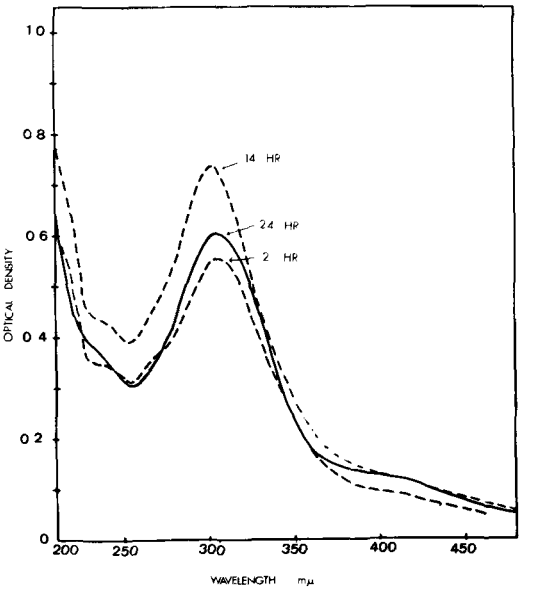

FIG. 4. Sulfuric acid absorption spectra of lipid A, after 24 hr, and of lipid B after 2 and 14 hr. Absorption maxima: both A and B, 303-305 nm.

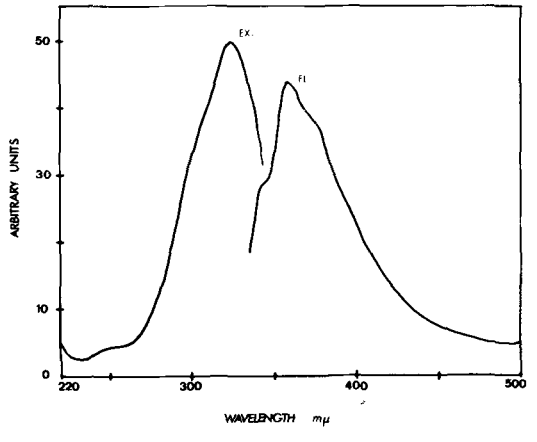

FIG. 3. Excitation and fluorescence spectra of lipid B in heptane. Excitation maximum, 320 nm; fluorescence maximum, 360 nm.

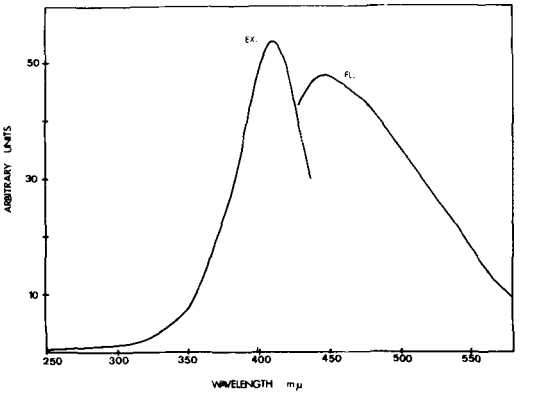

FIG. 5. Excitation and fluorescence spectra of lipid A in concentrated sulfuric acid. Excitation maximum, 405 nm; fluorescence maximum, 442 nm.

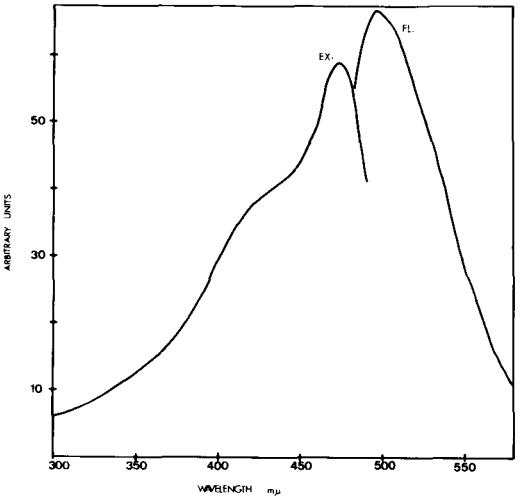

FIG. 6. Excitation and fluorescence spectra of lipid B in concentrated sulfuric acid. Excitation maximum, 473 nm; fluorescence maximum, 492 nm.

Observation of plates under UV light revealed a fluorescent band near the solvent front. In some experiments, special precautions were taken to avoid artefactual fluorescence from oxidized lipids. The chloroform-methanol extract was evaporated to a small volume in the cold under N_2 by solvent replacement (adding chloroform and reducing to a small volume as many times as necessary to replace methanol and water). In addition, the TLC plates were dried under N_2 (N_2 bag) after the completion of solvent development. The fluorescent lipid was observed as previously described after these precautionary measures.

The fluorescent band obtained in step one was scraped off as a 1 cm width of adsorbent layer. The lipids were eluted with chloroform by shaking the adsorbent gently in a centrifuge tube, and the adsorbent was sedimented in a clinical centrifuge. The eluent was removed and concentrated to a small volume under a stream of N_2, but was not dried.

The concentrated chloroform solution of the eluted lipid was applied to TLC plates in an even streak parallel to the lower edge of the plate. For the second step, the plates were developed in benzene-methanol-ethyl acetate (85:10:5 v/v) as described by Doorenbos and Sharma (4). In this case, the chamber was lined with paper and well saturated 12 to 24 hr before use. After development, two fluorescent bands were seen under UV light and scraped separately from the plates. Sometimes a second development in the same solvent system was necessary for clear separation of two distinct fluorescent bands.

Analytical Procedures

Absorption spectra in heptane and in sulfuric acid were determined in a Cary 14 recording spectrophotometer. The fluorescent lipids were studied in heptane because they were poorly soluble in ethanol, the standard solvent for establishing absorption characteristics. Excitation and fluorescence spectra were determined in heptane and sulfuric acid with a spectrofluorometer, Model SF-1 (Baird Atomic, Inc., Cambridge, Mass.), equipped with a Varian X-Y recorder. IR spectra were examined in a Perkin-Elmer Infracord Spectrometer; the sample was dissolved in chloroform and applied on KBr tablets. Because the fluorescent lipids had shown a rose color on heating with sulfuric acid, various other color reactions for steroid compounds were performed on thin layer plates with reagents prepared as described by Neher (5).

Reagents

Redistilled chloroform containing 1% ethanol as preservative was used; sulfuric acid was Baker Analyzed reagent grade; *n*-heptane was spectranalyzed reagent from Fisher Scientific Co.

RESULTS

Fluorescent lipids were recovered in the amount of about 0.1 mg/g wet weight of bovine brain white matter. On Silica Gel G thin layer plates, developed with benzene-methanol-ethyl acetate (85:10:5 v/v) as solvent in the second preparative step, two distinct fluorescent bands A and B were separated, with R_f values of 0.72 and 0.65, respectively. Wider separation was obtained by running the same plate a second time in the same solvent. A third faint fluorescent band migrated below band B. The quantity of material in this third band was much less and it was not studied. Between the A and B bands a yellowish UV absorbing band was seen that was not fluorescent. It was left behind when bands A and B were scraped from the plate. Other nonfluorescent bands were also evident by I_2 treatment of the plate.

The results of studies in heptane on the UV absorption of lipids A and B (Fig. 1) as well as their excitation and fluorescence spectra (Fig. 2 and 3) showed distinct resemblances between the two, but all maxima for A were at shorter wavelengths. After concentrated sulfuric acid treatment for 2-24 hr at 22 C, there was no difference between the absorption spectra of the two lipids (Fig. 4), but the excitation and fluorescence spectra of A (Fig. 5) had maxima at shorter wavelengths than did those of B (Fig. 6).

TABLE I

Characteristics of Bovine White Matter Lipids on Silica Gel G Thin Layer Plates

Spray reagents[a]	Daylight		Fluorescence in UV light	
	Lipid A	Lipid B	Lipid A	Lipid B
None	None	None	Bluish	Bluish
50% Sulfuric acid	Rose	Brown	Orange	Greenish-white
Liebermann-Burchard	Purple	+		
Phosphoric acid	Rose	Yellow-gray	Orange	Yellow
Silicotungstic acid	Purple	Yellow-brown		
$SbCl_3$ in $CHCl_3$	Yellow-gray	Gray		
2,4-Dinitrophenyl hydrazine	Yellow	+		
m-Dinitrobenzene (Zimmerman's test)	Violet-brown	Brownish		
Triphenyl tetrazolium Cl	Red	Red		
Isonicotinic acid hydrazide	None	None		
Phosphomolybdic acid	Blue	Blue		
Phosphotungstic acid	Yellow	Brownish	Orange	

[a]Spray reagents were prepared as described by Neher (5).

The material recovered from bands A and B showed, in both cases, a strong absorption peak in heptane in the region between 200 and 210 nm, in addition to the absorption peaks between 250 and 270 nm wavelengths.

IR absorption spectra of lipids A and B were identical except that B had an additional absorption at about 12.5 μ. The patterns were not diagnostic of any particular lipid and have not been included here.

Various color tests for steroid compounds (Table I) were positive except for the isonicotinic acid hydrazide reaction which specifically detects Δ1,4- and Δ4,3-ketosteroids.

DISCUSSION

Only one of the fluorescent lipids previously reported in brain has been identified. Geiger and Bampton (6) obtained a waxy fluorescent material from the brains of rabbits killed by diisopropyl fluorophosphate, but its presence in normal brain was not discussed; analyses were consistent with its being a fatty acid ester of the enol form of acetopiperone. Lunt and Rowe (7-9) found some fluorescent lipids that gave a color test suggesting a steroid nature in the neutral lipid fraction from mouse brain. The mouse brain lipids were isolated by a technique different from that used in the present study; the fluorescent lipids of mouse brain showed absorption peaks from 200-210 nm and from 250-270 nm; excitation and fluorescence spectra were not published. The IR absorption spectra of the lipids from mouse brain were unlike those from bovine white matter except for a peak at a wavenumber of about 2800 cm^{-1}. The chemical structure of the mouse brain fluorescent lipids has not been established.

Although the present fluorescent lipids of bovine white matter resembled steroids in respect to UV absorption, excitation and fluorescence spectra after treatment with concentrated sulfuric acid, and reaction to a number of color tests, all these methods are nonspecific. Further work will be needed to identify these minor components of the neutral lipid fraction of brain.

ACKNOWLEDGMENTS

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Isovaleroyl Triglycerides From the Blubber and Melon Oils of the Beluga Whale *(Delphinapterus leucas)*

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ABSTRACT

The fatty acid compositions of the blubber and melon oils from the beluga whale (*Delphinapterus leucas*) have been determined by gas liquid chromatography (GLC). The melon oil contains a high level (60.1 mole %) of isovaleric acid, substantial amounts of long chain branched acids (16.9%), and very little polyunsaturated material (0.5%). The blubber oil contains less isovaleric (13.2%), fewer long chain branched acids (2.7%), and appreciable amounts (10.9%) of the polyunsaturated acids typical of marine oils. The blubber and melon oils were also examined for lipid class composition by thin layer chromatography on silicic acid, direct GLC of the hydrogenated oil, and gel permeation chromatography. Both oils are composed almost entirely of triglycerides, which can be separated chromatographically into molecules containing 0, 1 and 2 isovaleric acid moieties. No triisovalerin could be detected. The blubber oil contains 68.9 mole % normal triacyl-, 24.2% diacyl-monoisovaleroyl-, and 7.0% monoacyl-diisovaleroyl-triglycerides (acyl = long chain acid). Monoacyl-diisovalerin constitutes 86.7 mole % of the melon oil. This unusual compound may play a role in the echolocation system of the beluga whale.

INTRODUCTION

Isovaleric acid has been reported in the melon (forehead), jaw and blubber oils of dolphins (1-4), porpoises (5-9), the pilot whale (10), and the beluga whale (11). Apparently only this small group of marine mammals out of the entire animal kingdom is able to biosynthesize fatty oils containing this unusual fatty acid. Recent biological studies by Norris (12,13) indicate that the fatty tissues in the melon and jaw of dolphins, porpoises and toothed whales (i.e., the suborder Odontoceti) may play a major role in the echolocation system of these animals. It seems appropriate, therefore, to conduct a detailed study of Odontoceti head oils to determine if their unusual chemical structure might possibly be related to their role in echolocation.

Lovern (7) and Varanasi and Malins (6) have reported that isovaleric acid occurs in mixed acid triglycerides of the melon, jaw and body oils from the porpoise *Phocoena phocoena*. On the other hand, Tsujimoto and Koyanagi (10) found both cetyl isovalerate and mixed isovaleroyl-long chain triglycerides in the head oil of the pilot whale *Globicephala macrorhyncha*. Varanasi and Malins (5) have also identified isovalerate wax esters in *Tursiops gilli* jaw oil. This paper reports a detailed comparison of molecular species of triglycerides found in the blubber and melon oils of the Arctic beluga whale, *Delphinapterus leucas*.

EXPERIMENTAL PROCEDURES

Materials

Beluga melon oil (*Delphinapterus leucas*) was supplied by Churchill Whale Products Ltd., Churchill, Manitoba. It came from the combined melons of several whales caught in Hudson Bay in August 1966. Beluga blubber oil was obtained from commercial production by the same company.

Gas Liquid Chromatography of Fatty Acids

Triglycerides were converted to methyl esters by heating in a screw-cap (Teflon lined) centrifuge tube with 5 ml 7% (w/v) BF_3/CH_3OH reagent per 100 mg as described by Morrison and Smith (14). Hexane rather than benzene was added to promote sample solubilization, since benzene was found to produce an artifact with a retention time on butanediolsuccinate polyester (BDS) columns very similar to that of methyl isobutyrate. Esters were normally concentrated into the hexane layer for gas liquid chromatography (GLC) analysis by the addition of 1.5 ml water to the reaction tube. Occasional direct GLC study of homogeneous reaction mixtures was attempted to check the quantita-

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TABLE I

Fatty Acid Composition of the Blubber and Melon Oils From the Beluga Whale

Fatty acid		Blubber oil		Melon oil	
		Weight %	Mole %	Weight %	Mole %
Saturated					
Iso	5:0	5.80	13.15	40.57	60.09
	9:0	---	---	0.10	0.10
Iso	10:0	0.03	0.05	0.08	0.07
	10:0	0.10	0.13	0.25	0.22
Iso	11:0	0.10	0.13	1.38	1.19
Anteiso	11:0	0.03	0.05	Trace	Trace
	11:0	0.05	0.08	0.06	0.05
Iso	12:0	0.10	0.13	0.56	0.45
	12:0	1.00	1.24	1.29	1.03
Iso	13:0	0.25	0.32	2.50	1.89
Anteiso	13:0	0.03	0.03	Trace	Trace
	13:0	0.08	0.11	0.10	0.07
Iso	14:0	0.29	0.32	1.79	1.27
	14:0	6.81	7.39	6.81	4.83
4,8,12-Trimethyltridecanoic		0.31	0.34	---	---
Iso	15:0	0.64	0.66	14.64	9.80
Anteiso	15:0	0.10	0.11	0.48	0.33
Unknown		---	---	0.21	0.14
	15:0	0.33	0.34	0.54	0.36
Iso	16:0	0.17	0.16	2.06	1.31
Pristanic		0.12	0.10	---	---
	16:0	8.41	8.18	5.53	3.53
Iso	17:0	0.18	0.16	0.62	0.38
Anteiso	17:0	0.08	0.08	0.13	0.09
	17:0 Phytanic	0.52	0.47	0.39	0.24
Iso	18:0	0.07	0.05	0.01	0.01
	18:0	1.51	1.34	0.17	0.12
	19:0	Trace	Trace	Trace	Trace
	20:0	0.04	0.03	0.07	0.03
Total saturated		27.15	35.15	80.34	87.60
Monounsaturated					
	12:1	0.39	0.47	0.24	0.19
	14:1ω?	0.38	0.47	0.39	0.28
	14:1ω?	0.50	0.55	0.39	0.28
	14:1ω?	0.76	0.84	0.39	0.28
	15:1	Trace	Trace	0.49	0.33
	Unknown	0.63	0.60	0.24	0.15
	16:1ω9	1.11	1.08	1.09	0.71
	16:1ω7	20.12	19.73	10.95	7.02
	16:1ω5	0.31	0.32	0.09	0.05
	17:1ω8	0.13	0.13	0.04	0.02
	18:1ω11+9	15.57	13.81	2.96	1.72
	18:1ω7	4.29	3.81	0.62	0.36
	18:1ω5	0.60	0.53	0.28	0.15
	19:1ω9	0.01	0.01	---	---
	19:1ω8	0.01	0.01	---	---
	19:1ω7	0.03	0.03	---	---
	20:1ω11+9	9.32	7.53	0.52	0.28
	20:1ω7	0.58	0.47	0.03	0.02
	20:1ω5	0.04	0.03	Trace	Trace
	22:1ω13+11	3.42	2.55	0.12	0.05
	22:1ω9	1.07	0.79	0.02	0.02
	22:1ω7	0.12	0.08	---	---
	24:1ω9	0.21	0.16	---	---
Total monounsaturated		59.60	54.00	18.86	11.91

(Continued on following page)

TABLE I
(Continued from following page)

Polyunsaturated				
16:2ω6	0.03	0.03	0.04	0.03
16:2ω4	0.33	0.32	0.13	0.08
16:3ω4	0.03	0.02	—	—
16:3ω3	0.09	0.08	0.02	0.02
16:4ω3	0.17	0.16	0.08	0.05
16:4ω1	0.12	0.13	0.07	0.05
18:2ω6	1.43	1.29	0.19	0.10
18:3ω6	0.05	0.05	0.02	0.02
18:3ω3	0.14	0.13	0.02	0.02
18:4ω3	0.25	0.24	—	—
20:2ω6	0.10	0.08	0.03	0.02
20:3ω6	0.01	Trace	—	—
20:3ω3	Trace	Trace	—	—
20:4ω6	0.33	0.26	0.03	0.02
20:4ω3	0.30	0.24	—	—
20:5ω3	3.88	3.24	0.16	0.08
21:5ω2(?)	Trace	Trace	—	—
22:4ω6	Trace	Trace	—	—
22:5ω6	Trace	Trace	—	—
22:5ω3	1.91	1.45	—	—
22:6ω3	4.08	3.13	—	—
Total polyunsaturated	13.25	10.85	0.79	0.49
	100.00	100.00	99.99	100.00

tion of the C_8-C_{12} acids.

The apparatus employed for GLC determination of all longer chain length fatty acids was a Perkin-Elmer Model 226 gas chromatograph fitted with a flame ionization detector (FID) and a stainless steel, open tubular (capillary) column 50 m long and 0.25 mm i.d. The coating was BDS polyester. Standard operating conditions were: column, 170 C; injection port, 270 C; helium carrier gas, 40 psig (giving a flow rate of 5 ml/min at 25 C). For study of lower molecular weight materials, an initial period of isothermal operation at 90 C was followed by a rapid temperature rise after emergence of the 10:0 peak and subsequent operation under standard conditions. This permitted accurate qualitative assessment of materials with chain lengths as low as C_4; but quantitation of C_4-C_6 material in relation to higher C_{16}-C_{18} fatty acid methyl esters was erratic, and the important isovaleric acid component was therefore determined independently (see below). Peaks were identified by comparison with the retention times of known standards and by graphical analysis of their retention times as previously described (15). Peak areas were determined using a Disc Instruments integrator and were corrected to weight per cent composition as described by Ackman and Sipos (16) for the FID. This data was subsequently integrated with an independent isovaleric acid determination and converted to mole per cent.

Determination of Isovaleric Acid

Isovaleric acid was determined by GLC using the internal standard technique. After qualitative verification of the absence of valeric acid (none was detected in beluga samples), about 100 mg of blubber or melon oil was weighed into a 100 ml volumetric flask. Five milliliters of 0.5 N KOH in *n*-butanol (BuOH) containing an appropriate amount of valeric acid standard was added. The mixture was heated on a steam bath for 15 min, with occasional swirling, and cooled. Five milliliters of 14% (w/v) BF_3/BuOH reagent was added, followed by a further heating for 15 min to esterify the liberated acids. After cooling, distilled water was added to volume and the mixture shaken briskly. Part of the BuOH layer (3-5 ml) was transferred to a 100 ml volumetric flask, and a saturated NaCl solution with approximately 0.25 ml petroleum ether was added to bring the ester rich layer which separated after shaking into the narrow neck of the flask for convenient removal by syringe for GLC analysis. GLC analyses were carried out on a glass column, 2 m x 3 mm i.d., packed with 5% SE-30 on 70/80 mesh Anakrom ABS. The apparatus was a Barber-Colman Model 10, fitted with FID, operated at a column temperature of 120 C and a carrier gas pressure of 20 psig. Peak areas were determined with a Disc Instruments integrator. The isovaleric acid content was then calculated from the ratio of the 5:0 and iso-5:0 peak areas, the amount of 5:0 added, and the weight of the original sample.

Since the triglyceride fractions recovered from preparative thin layer chromatography (TLC) separations were too small to be weighed

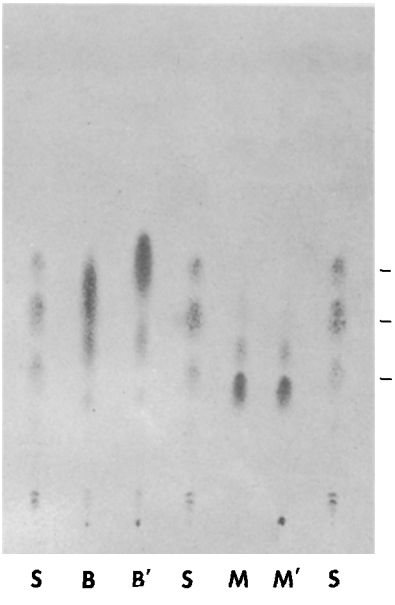

FIG. 1. Separation of beluga blubber and melon triglycerides according to isovaleric acid content using TLC on silicic acid. XXX, triglycerides containing no isovaleric acid; XXV, diacyl-monoisovalerin; XVV, monoacyl-diisolvalerin; S, standard mixture of XXX, XXV, XVV and VVV (not marked) produced by interesterification of trimyristin and triisovalerin. B, unhydrogenated beluga blubber oil. B', hydrogenated beluga blubber oil. M, unhydrogenated beluga melon oil. M', hydrogenated beluga melon oil. Operating conditions: 200 x 200 mm TLC plate coated with 0.25 mm layer of Adsorbosil 1; developed in 87:12:1 petroleum ether-diethylether-acetic acid; spots visualized by charring with $H_2SO_4/K_2Cr_2O_7$ solution.

accurately, they were analyzed using both valeric and heptadecanoic acid internal standards permitting complete quantitation in a step-programmed GLC analysis. From 1-4 mg of sample were placed in a 50 ml volumetric flask and 1.0 ml 0.5 N KOH/BuOH containing the appropriate amounts of acid standards was added. The stoppered flask was heated on a steam bath for 10 min, and then 1.0 ml 14% (w/v) BF_3/BuOH reagent was added. The restoppered flask was heated for another 10 min, cooled, and filled to 50 ml with distilled water. Then 0.25 ml petroleum ether was added and the flask shaken vigorously for 1 min. After separation, a sample for GLC analysis was taken directly from the top layer. GLC analysis of the C_5 components was carried out as before, following which the column temperature was rapidly raised to 200 C for the rest of the analysis. The total fatty acid composition of the sample was then calculated from the ratio of the 5:0 and iso-5:0 peak areas, the ratio of the 17:0 and C_{10}-C_{22} peak areas, and the amounts of 5:0 and 17:0 originally added.

TABLE II

Isovalerate Content of the Triglyceride Bands Separated From Hydrogenated Beluga Blubber Triglycerides by Preparative TLC

Triglyceride bands	Mole % isovalerate
Band XXX[a]	Trace
Band XXV	30.2
Band XVV	61.0

[a]X, long chain fatty acid; V, isovaleric acid.

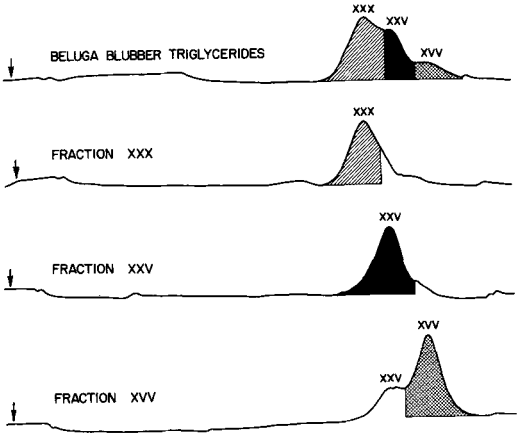

FIG. 2. Separation of unhydrogenated beluga blubber oil triglycerides by permeation chromatography. Initial bands from total sample (top curve) were individually rechromatographed (three bottom curves) for further purification. Shaded areas represent fractions collected. XXX, triglycerides containing no isovaleric acid; XXV, diacyl-monoisovalerin; XVV, monoacyl-diisovalerin. Operating conditions: 25 x 420 mm column of 200/400 mesh styrene divinyl-benzene copolymer beads; ∿ 100 mg triglycerides dissolved in 1.0 ml benzene applied to column; samples eluted with benzene at 1.3 ml/min; column temperature 28 C.

Although a very small amount (∿0.6 mole %) n-C_{17} acid was present in the original blubber triglycerides, this method was quite accurate enough to identify the fractions of triglycerides containing no isovaleric acid (XXX), diacyl-monoisovalerin (XXV), and monoacyl-diisolvalerin (XVV) separated by TLC.

Hydrogenation

To improve resolution in TLC and GLC, both melon and blubber oils were fully hydrogenated prior to triglyceride analysis. This was accomplished by the method of Farquhar et al. (17) using freshly distilled dioxane as a solvent in place of ethanol.

TLC

Hydrogenated beluga blubber and melon lipid

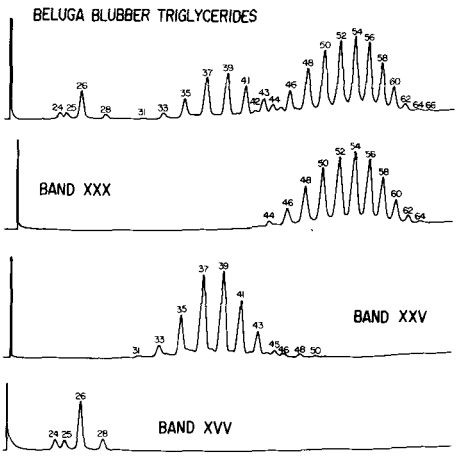

FIG. 3. Gas chromatograms of hydrogenated total beluga blubber triglycerides and of the three bands separated by TLC on silicic acid. XXX, triglycerides containing no isovaleric acid; XXV, diacyl-monoisovalerin; XVV, monoacyl-diisovalerin. Operating conditions: 560 x 2.5 mm i.d. stainless steel column packed with 3.0% JXR on 100/120 mesh Gas Chrom Q; column temperature programmed 160→350 C at 4 deg/min; 100 ml/min. He carrier gas; flash heater 350 C.

were separated by preparative TLC on 1.0 mm thick layers of silicic acid (Adsorbosil 1, Applied Science Laboratories, State College, Pa.) impregnated with 0.02% Rhodamine 6G. Plates were developed in 87:12:1 petroleum ether-diethylether-acetic acid. Bands were then located under UV light, scraped into individual chromatography columns, and eluted with diethylether.

Permeation Chromatography

The triglycerides from 1.0 g of unhydrogenated beluga blubber oil were eluted from a 20 x 100 mm column of alumina (Woelm, basic activity grade 1) with 150 ml diethylether. After solvent evaporation, the triglycerides were redissolved in 10 ml benzene and applied, 1 ml of solution at a time, to a 25 x 420 mm styrene-divinylbenzene copolymer bead column (Dow X2, 200/400 mesh, lot 07153). The sample was eluted with additional benzene at 28 C with a flow rate of 1.3 ml/min (18). A differential recording refractometer (19) was used to monitor the eluate. The respective triglyceride fractions from the 10 individual runs were combined and chromatographed once more on the same column for further purification.

GLC of Triglycerides

GLC analyses of hydrogenated triglycerides were carried out under the optimum operating conditions for marine oils described by Harlow, Litchfield and Reiser (20). An F & M 400 gas chromatograph equipped with FID and automatic temperature programming was used. The 560 x 2.5 mm i.d. stainless steel column packed with 3.0% JXR on 100/120 mesh Gas Chrom Q was programmed from 160 C to 350 C at 4 deg/min with 100 ml/min He carrier gas.

Peaks were identified as to carbon number by cochromatography with known compounds. Trimyristin, tripalmitin, tristearin, triarachidin

TABLE III

Triglyceride Composition of Hydrogenated Blubber and Melon Oils From the Beluga Whale

Carbon no.	Blubber oil, mole %	Melon oil, mole %
Diisovaleroyl triglycerides (XVV)		
20	---	0.17
22	---	1.27
23	—	3.89
24	1.24	18.07
25	1.30	31.78
26	3.78	28.66
27	—	0.60
28	0.64	2.27
Total XVV	6.96	86.71
Monoisovaleroyl triglycerides (XXV)		
29	—	
31	0.29	
33	0.86	
34	0.28	10.49
35	2.68	
36	0.53	
37	5.64	
38	0.31	—
39	5.86	0.95
40	0.17	—
41	4.17	0.30
43	2.33	0.19
45	0.86	0.13
47	0.20	0.11
Total XXV	24.18	12.17
Triglycerides with no isovaleric acid (XXX)		
42	0.51	Trace
44	1.41	0.08
46	3.37	0.17
48	6.43	0.27
50	9.06	0.21
52	10.69	0.16
54	11.03	0.11
56	10.31	0.07
58	7.93	0.04
60	4.96	—
62	2.23	—
64	0.76	—
66	0.17	—
Total XXX	68.86	1.11
	100.00	99.99

and tribehenenin of 99% purity (Applied Science Laboratories, State College, Pa.) were used to identify the carbon numbers of triglycerides containing no isovaleric acid. Mixtures of tristearin and triisovalerin (Eastman Organic Chemicals, Rochester, N.Y.), tripalmitin and triisovalerin, and trimyristin and triisovalerin were interesterified with a $NaOCH_3$ catalyst (21) to produce standards of isovaleroyl-triglycerides. Interesterification of trimyristin and triisovalerin, for example, produced a mixture of trimyristin, isovalero-dimyristin, diisovalero-myristin and triisovalerin that was used to identify the peaks of 15, 24, 33 and 42 carbon number. Peak areas were determined by planimetry. Quantitative response factors for the various carbon number triglycerides were determined from known composition mixtures of trihexanoin, trioctanoin, tridecanoin, trilaurin, trimyristin, tripalmitin, tristearin, triarachidin and tribehenin. The f_m values (molar calibration factors determined by the internal normalization technique) (22) of the simple triglycerides in the calibration mixtures were then plotted vs. carbon number, and f_m values for all carbon numbers were interpolated from the resulting graph. Triglyceride compositions are reported in mole per cent.

RESULTS AND DISCUSSION

Fatty Acid Composition

Since beluga blubber and melon oils are known to contain isovaleric acid, the first step was to determine the general fatty acid composition of our samples. GLC analysis of the fatty acids gave the results reported in Table I. The melon oil contains a very high level (60.1 mole %) of isovaleric acid, substantial amounts of long chain branched acids (16.9%), and very little highly polyunsaturated material (0.5%). The blubber oil contains less isovaleric (13.2%), fewer long chain branched acids (2.7%), and appreciable amounts (10.9%) of the polyunsaturated acids typical of marine oils.

The types and amounts of long chain branched acids in the blubber oil are similar to those found in most marine depot lipids (23,24); only in the melon oil do they accumulate to an unusual degree. Most of the long chain branched acids are of the *iso* series, indicating their possible genesis from isovaleric acid. The levels of isovaleric acid reported here for beluga melon and blubber oils are almost twice those found by Williams and Maslov (11). Assuming their analyses to be reasonably accurate, it would appear that the isovaleric acid content of the beluga whale is subject to considerable variation. Although of circumpolar distribution, the genus *Delphinapterus* has but a single species, so this difference in isovaleric content cannot be attributed to species differences.

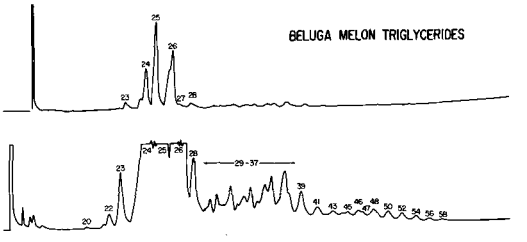

FIG. 4. Gas chromatograms of hydrogenated total beluga melon triglycerides. Column was overloaded in lower chromatogram so that minor peaks could be quantitated. Operating conditions same as for Figure 3.

TLC

Beluga melon and blubber oils were next examined for lipid class composition by TLC on silicic acid. Both samples could be separated into three main spots in an 87:12:1 petroleum ether-diethylether-acetic acid solvent system (Fig. 1). Cochromatography with trimyristin, isovalero-dimyristin, diisovalero-myristin and triisovalerin showed that these three spots correspond to triglyceride molecules containing 0, 1 and 2 isovaleroyl moieties. The greatest distance up the plate was covered by XXX; XXV moved somewhat less; while XVV moved the shortest distance. No triisovalerin could be detected by TLC. Resolution of the three spots was significantly improved by hydrogenation of the samples prior to TLC.

Further evidence for the identify of the isovaleroyl-triglycerides was obtained by recovering the XXX, XXV, and XVV bands from a preparative TLC separation of hydrogenated beluga blubber oil and determining the fatty acid composition of each fraction. The results (Table II) show the expected compositions. Fraction XXX contains essentially no isovaleric acid; fraction XXV contains about one third isovaleric acid on a molar basis; and fraction XVV is approximately two thirds isovaleric. Slight deviations from theoretical isovalerate content are probably due to incomplete separation of the three bands.

The resolution of triglycerides containing 0, 1, 2 and 3 isovaleric acid moieties is not completely unexpected. Other workers such as Blank and Privett (25) and Kleiman et al. (26) have shown that triglycerides containing one acetic or butyric acid moiety can be easily separated from long chain triglycerides by TLC on silicic acid. Certainly this type of separation

is extremely useful with cetacean triglycerides containing isovaleric acid, since it allows rapid, easy fractionation of these complex mixtures according to isovaleric acid content.

Permeation Chromatography

Resolution of beluga blubber triglycerides by permeation chromatography on a polystyrene gel column yielded three fractions (Fig. 2). After rechromatography for further enrichment, GLC of the intact triglycerides from each fraction clearly identified them as the XXX, XXV and the XVV bands already found by TLC. This resolution of XXX, XXV and XVV triglycerides by permeation chromatography would generally be expected, since this technique separates molecules mainly on the basis of molecular weight (27). The resolution of the XXX, XXV and XVV fractions by permeation chromatography is approximately the same as by TLC on silicic acid, provided sample size and enhancement of resolution by hydrogenation are considered.

GLC

Natural triglycerides mixtures containing short chain fatty acids can be fractionated into a large number of peaks by GLC (28,29). Figure 3 (top) shows the resolution of the hydrogenated blubber oil triglycerides by this technique into a phenomenal 30 peaks. These peaks seem to be arranged into three groupings: C_{24}-C_{28}, C_{31}-C_{45} and C_{42}-C_{66}. The identities of these three groups of beluga blubber triglycerides were clarified by GLC analysis of the XXX, XXV and XVV bands that had been isolated from hydrogenated beluga blubber triglycerides by preparative TLC. Band XXX contains only C_{42}-C_{66} triglycerides of even carbon number (Fig. 3) corresponding to triglyceride molecules containing no isovaleric acid. Band XXV contains mostly odd carbon number C_{31}-C_{45} peaks, which are triglycerides having one isovaleroyl moiety per molecule. Band XVV contains C_{24}-C_{28} material, which must be monoacyl-diisovalerins. Thus, these chromatograms provide additional evidence for the identity of the XXX, XXV and XVV bands tentatively identified by their TLC R_f values and their fatty acid compositions. The large differences in triglyceride retention times attributable to the presence of 0, 1 or 2 isovaleric acid chains allow complete GLC quantitation of the XXX, XXV and XVV fractions without prior subfractionation by TLC on silicic acid. XXV molecules are mainly odd carbon number triglycerides and thus have different elution temperatures from the XXX species, which are mostly even carbon number triglycerides. Quantitation of the chromatograms (Table III) shows that beluga blubber oil contains 68.9 mole % XXX, 24.2% XXV, and 7.0% XVV. No triisovalerin could be detected by GLC, even when the starting column temperature was lowered to 100 C.

GLC analysis of hydrogenated beluga melon triglycerides (Fig. 4) shows them to be composed of mostly XVV species from C_{23} through C_{28}. Injection of an extra large sample indicates that XXV and XXX species are indeed present but only in minor amounts. No peak was observed at the temperature where synthetic triisovalerin eluted from the column. Monoacyl-diisovalerin constitutes 86.7 mole % of the melon triglycerides (Table III); very little XXV (12.2%) and XXX (1.1%) are present. The high level of branched-chain acids in beluga melon triglycerides leads to shouldering on many of the peaks. Note that the C_{24} and C_{26} peaks both exhibit front shoulders, probably due to the presence of both iso- and n-long chain acids in the XVV molecules. These shoulders could also be due to the presence of triglyceride positional isomers (29), but the presence of high levels of iso-14:0 and iso-16:0 acids makes the former explanation considerably more probable. The C_{29}-C_{37} area of the beluga melon triglycerides is extremely complex on the chromatogram in Figure 4. The presence of both short and long chain branched acids in the same XXV molecules probably accounts for this complexity. Thus accurate carbon number assignments could not be made for the C_{29}-C_{37} peaks, and they were quantitated as a single group for reporting in Table III.

Comparison of average fatty acid chain length values (20) indicates that the carbon number distributions reported in Table III are quite close to the correct values. The triglyceride data for the blubber oil gives an average acyl chain length of 15.99 vs. 15.63 calculated from the fatty acid composition. The respective values for the melon oil are 8.85 and 9.01.

Implications for Cetacean Echolocation

Norris (12,13) and Wood (30) have postulated that the melon of fatty tissue in the forehead of the beluga whale and other Odontoceti (dolphins, porpoises and toothed whales) functions as a sound transducer and possibly a sonic lens in the echolocation process of these animals. The sound transmitter for echolocation apparently lies in the nasal passages, immediately behind the melon. This sound is picked up by the fatty tissue in the melon and directionally transmitted to the seawater in front of the head. A fatty oil is closely

impedance-matched to seawater so that the energy losses during the transfer of sound waves from melon to seawater are very low (13). The present work indicates that a triglyceride, monoacyl-diisovalerin, constitutes 86.7 mole % of the melon oil in the beluga whale. The long chain acyl moiety of this triglyceride is primarily iso and straight chain C_{11}-C_{17} fatty acids. Many animals other than ruminants probably produce minor amounts of isovaleric acid. Human blood, for example, contains about 194 μg/100 ml plasma (31). The Odontoceti are the exceptional animals which exploit this acid by incorporating it into their head and blubber oils. The presence of the acid in the blubber oils is possibly due to spillover of the triglycerides synthesized for the echolocation system in the head.

Gilmore (32) has suggested that isovaleric acid is needed to maintain the melon fatty oil in a liquid state at the extremely low environmental temperatures (down to -2 C) in which these animals live. The low temperature requirement is possibly due to a paucity of blood vessels in the skin overlying the melon, as those might interfere with high frequency sound transmission into the water, and a temperature gradient may exist across the melon. Ordinary marine oils, including those of many fish and of the Mysticeti, are subject to formation of solids (stearine) at temperatures in some cases as high as 20 C (discussed technically as cold clearing; cf. AOCS cloud point test) (33). In our experience beluga melon oil is clear down to well below 0 C.

It seems premature to advance any hypothesis relating lipid composition to echolocation ability in the Odontoceti until we have considerably more information on the precise melon and jaw lipids found in these animals as well as what unusual acoustical properties, if any, these isovaleric-containing oils might possess. Further experiments along these lines are currently in progress.

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SHORT COMMUNICATIONS

Sphingolipid Biosynthesis by Particulate Fractions of Normal and "Quaking" Mouse Brain

ABSTRACT

No differences were detected in the ability of brain particulate fractions isolated from "Quaking" and normal mice for the incorporation of: ^{14}C-L-serine into 3-keto-dihydrosphingosine; 1-^{14}C-stearoyl acid and 1-^{14}C-stearoyl CoA into ceramide; and ^{14}C-choline from CDP ^{14}C-choline into sphingomyelin.

The "Quaking" strain of mouse has been characterized genetically and histologically (1) and shown to bear an autosomal recessive gene (qk) which is responsible for abnormalities in central nervous system structure and function. The major neuropathological findings in this mutant animal is a generalized deficiency of myelin. Quantitative analysis of brain tissue from these animals have indicated marked decreases in cerebroside and sulfatide content as well as the long chain fatty acids characteristically associated with these lipids (2-4,6,7). Gangliosides, which are predominantly extramyelin, are present in quantities comparable to control animals. Administration of radioactive lipid precursors in vivo have indicated a diminished ability for synthesis of cerebral sphingolipids (8,9). In vitro studies have demonstrated dimunition of several enzymes which include: cyclic 2',3' AMP phosphohydrolase (10), a myelin marker; arylsulfatase A and α-mannosidase (11), two lysosomal enzymes; ceramide: UDP-galactose-galactosyl transferase (8,12,13; also, P. Morrell and N.S. Radin, personal communication) presumably responsible for ceramide galactoside biosynthesis.

This report documents the ability of particles isolated from brain tissue of "Quaking" animals to catalyze the synthesis of 3-keto-dihydrosphingosine, ceramide and sphingomyelin.

Quaking mutants and their congenic controls (C57BL165) 15-30 days old were obtained either from Jackson Laboratory, Bar Harbor, Maine, or from our own colony. Brain tissue was removed, homogenized and both 0-10,000 xg and 10-30,000 xg particles were prepared as previously described (14). U-^{14}C-L-Serine (S.A. =117 μc/μmole) 1-^{14}C-stearic acid (S.A.=38.2 μc/μmole), 1-^{14}C-stearoyl CoA (S.A.=46.08 μc/μmole) were obtained from New England Nuclear Corp., CDP-choline-^{14}C (S.A.=9.3

TABLE I

Conversion of Radioactive Precursors to Several Sphingolipids by Mouse Brain Particles

	Micro moles per milligram of protein
A. L-Serine-^{14}C→3-keto-dihydrosphingosine	
Normal	20
Quaking	29
B. CDP-choline-^{14}C→sphingomyelin	
Normal, no additions	6
Normal + 0.2 μmoles each *threo* sphingosine and palmitoyl CoA	8
Quaking, no additions	6
Quaking + 0.2 μmoles each *threo* sphingosine and palmitoyl CoA	11
C. 1-^{14}C-Stearoyl acid→ceramide	
Normal	0.9
Quaking	0.6
D. 1-^{14}C-Stearoyl CoA→ceramide	
Normal	23
Quaking	21

$\mu c/\mu mole$) was purchased from Tracerlab-INC Corp. Analtech Silica Gel G (250 μ) precoated thin layer plates were used. *Erythro* D L sphingosine was purchased from Miles-Yedda.

The incorporation of choline-^{14}C from CDP-choline-^{14}C into sphingomyelin was assayed as previously described (14) using the 0-10,000 xg particulate fraction. The palmitoyl CoA dependent condensation with L-serine-^{14}C into 3-keto-dihydrosphingosine by the 10-30,000 xg pellet was quantitated according to a published procedure (15). The *erythro*-sphingosine dependent biosynthesis of ceramide was assayed using 1-^{14}C-stearoyl CoA incorporation according to Scribney (16) and with 1-^{14}C-stearic acid by the method of Yavin and Gatt (17). The products were further purified by TLC (C-M-HOAc 94:1:5) to separate the radioactive ceramide from traces of 1-^{14}C-stearic acid which was present in the final extraction. Areas of the plate corresponding to ceramide standard were scraped and counted with Aquesol in a liquid-scintillation spectrometer.

The capacity for the enzyme preparations from the mutant animals to catalyze the palmitoyl CoA-dependent condensation of L-serine-^{14}C to form 3-keto-dihydrosphingosine is documented in Table IA. It is evident that tissues from both groups of animals are equally effective.

No deficiency was found in the ability of the mutant animals to carry out the incorporation of choline-^{14}C from CDP choline-^{14}C into sphingomyelin (Table IB). The mild alkaline and mild acidic-$HgCl_2$ hydrolysis (15) treatment were required for these studies due to the extensive formation of both phosphatidyl and phosphatidal choline.

Ceramide biosynthesis was assayed by the *erythro*-sphingosine catalyzed incorporation of both 1-^{14}C-stearic acid and 1-^{14}C-stearoyl CoA into this lipid and these results are presented in Table IC and D. It is apparent that there is no deficiency in the synthesis of this compound in the "Quaking" animals.

The data presented in Table I were derived from pooled brains from 20-day-old normal and "Quaking" animals. Although similar results were found with both younger and older animals, the absolute activities obtained were different. This age dependent variation in enzyme activity related to sphingolipid metabolism has been previously noted.

The in vitro studies reported here demonstrate that brain particles from the "Quaking" animals have no deficiency in catalyzing the biosynthesis of 3-keto-dihydrosphingosine, ceramide and sphingomyelin. 3-Keto-dihydrosphingosine is believed to be the initial substrate required for the eventual assemblage of the sphingolipids (18-20), while ceramide is the reported lipid acceptor molecule involved in galactocerebroside (21), glucocerebroside (22) and sphingomyelin biosynthesis (23). Although glucocerebroside is present in trace quantities in the central nervous system, it is believed to be a precursor of the gangliosides (24). The presence of the enzymes responsible for the synthesis of 3-keto-dihydrosphingosine and ceramide may be a reflection of the animals' requirements for ganglioside formation.

Clinically the "Quaking" animal is recognized by the 12th day post-partum by an unsteady gait and tremor of the hind quarters (1).Tonic-clonic seizures are readily induced by sensory stimulation. All "Quaking" animals employed in these studies displayed these symptoms. Several laboratories have reported a deficiency of ceramide UDP galactose-galactosyl transferase activity in "Quaking" animals (8,11,12; also, P. Morell and N.S. Radin, personal communication). In our experience a significant number of animals, approximately 15-20%, displayed the characteristic clinical symptoms but did not exhibit either a deficiency in this enzyme nor a decreased cerebroside content in whole brain tissue. The data reported in the present studies were derived only from those animals which had a reduction in both this enzymatic activity as well as cerebroside concentration. It is therefore suggested that "Quaking" animals, in addition to the usual clinical symptoms, be evaluated biochemically be investigators employing these animals for research purposes.

Ceramide galactoside-β-galactosidase activity was identical in both groups of animals in accord with the previous observations of Bowen and Radin (25). In addition, no differences between "Quaking" and normal animals were found in ceramide glucoside-β-glucosidase.

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Total Fatty Acids of Habituated and Teratoma Tissue Cultures of Tobacco[1]

ABSTRACT

Significant differences in the total fatty acids of habituated and teratoma tissue cultures of tobacco were noted. The teratoma tissues contained 3.2 times more total fatty acids than the habituated, while both tissues differed considerably from the fatty acids of tobacco seedlings used for comparison. Differences in relative concentration for each of the three tissues were also noted.

Habituated (normal origin) and teratoma (tumorous origin) tissues of tobacco grown in germ-free culture have been extensively described and characterized by Braun (1). Tissue cultures of these types have proven to be valuable tools for biochemical and physiological research with particular interest in phytosterol biosynthesis (2-5) and pigment-phytohormone relationships (6-7). With the recent interest in the composition and synthesis of aliphatic hydrocarbons of tobacco tissue cultures (8), the fatty acid distributions of these tissues are of importance. This communication reports the comparative total fatty acid levels of habituated and teratoma tissue cultures of tobacco. The total fatty acids of tobacco seedlings were used as reference materials for comparison.

The qualitative total fatty acid distributions of the habituated, teratoma and seedling tissues (Table I) were almost identical and typical of most higher plants previously reported (9). The fatty acids contained predominantly even-numbered carbon chains while odd-numbered chains were present in low concentrations. No branched chain fatty acids were detected, which is in contrast to a previous report by Mold et al. (10). They found low concentrations of branched and cyclohexyl isomers of normal acids in flue-cured tobacco. These compounds may very well be present in the teratoma and seedling tissues of this study, but were not detected using the quantities of tissue available. The habituated tissues would not, however, be expected to contain branched chain acids, since no branched chain alkanes were present in these same tissues (8). The major saturated acid in each tissue was palmitic, while linoleic and linolenic were the predominant unsaturated acids (Table I). The relative concentration ratios of the unsaturated C_{18}

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TABLE I

Total Fatty Acid Composition of Habituated, Teratoma and Seedling Tissues of Tobacco[a]

Fatty acid	Tissue culture		
	Habituated, %	Teratoma, %	Seedling, %
C_{14}	—	Trace	Trace
$C_{14:1}$	—	Trace	Trace
C_{15}	1.2	1.0	—
C_{16}	15.1	24.5	11.4
$C_{16:1}$	0.1	1.9	2.4
C_{17}	1.4	1.2	0.8
C_{18}	3.3	6.0	1.8
$C_{18:1}$	16.4	8.3	7.2
$C_{18:2}$	35.3	33.7	17.5
$C_{18:3}$	32.1	27.2	57.2
C_{20}	0.9	0.8	0.6
C_{21}	Trace	Trace	0.1
C_{22}	Trace	Trace	0.2
$C_{22:1}$	—	—	Trace
C_{24}	—	0.6	Trace
Total fatty acids, mg/g dry weight	0.40	1.31	42.03
Unsaturated-saturated acid ratio	3.81	2.09	5.66

[a]Each value represents a percentage of the total fatty acids.

acids ($C_{18:1}$, $C_{18:2}$, $C_{18:3}$) of the habituated, teratoma, and seedling tissues were approximately 1:2:2, 1:4:3 and 1:2:8, respectively. Thus, with respect to these acids, higher unsaturation with increased differentiation capacity seems to be the tendency (habituated < teratoma < seedling). This is only partially true when considering the unsaturated to saturated acid ratio (U/S) of each tissue (Table I). The high unsaturation of the seedling tissues is again illustrated while the U/S ratio of habituated tissues indicates higher unsaturation levels than the teratoma tissues (teratoma < habituated < seedling).

Significant differences in total fatty acid concentrations were also noted. Habituated and teratoma tissues differed in fatty acid concentration by a factor of 3.2 (teratoma > habituated) while considerably higher levels were found in the seedling tissues (Table I). When compared, habituated tissues generally contains lower concentrations of chemical constituents and lower metabolic activity than teratoma tissues (2,3,5). It has been suggested that this is due to the low living to dead cell ratio or to the low cellular cytoplasm to vacuole ratio of the callus, or both (J.D. Weete and C.H. Walkinshaw, submitted for publication).

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Determination of *Cis* and *Trans* in Monoene and Diene Fatty Esters by Gas Chromatography

ABSTRACT

A GC method has been developed for quantitative determination of the *cis* and *trans* content in monoene and diene C_{18} fatty esters. In order to achieve separation of the *cis*- and *trans*-monoene and diene isomers, the fatty esters are first epoxidized with peracetic acid. The epoxidation procedure is simple and stereoselective. Positional and geometric isomerization of the double bond does not occur. The GC analysis requires no exotic equipment, reagents or techniques and utilizes standard columns packed with EGSS-X. Satisfactory analyses were obtained for synthetic mixtures containing oleate-elaidate and *trans,trans*-, *cis,trans*- and *cis,cis*-dienes.

We wish to report a simple gas chromatographic procedure for determining *cis* and *trans* percentages in monoene and diene fatty esters. The procedure utilizes peracid epoxidation of olefinic bonds which is an established stereospecific reaction (1). The *trans* olefins yield *trans* epoxide derivatives and *cis* olefins form *cis* epoxides. Epoxidation of methyl oleate with peracetic acid produces methyl *cis*-9-epoxystearate in about 97% yield (2). Epoxidation of methyl linoleate is also reported to give over 95% yield of methyl *cis*-9,*cis*-12-diepoxystearate (3).

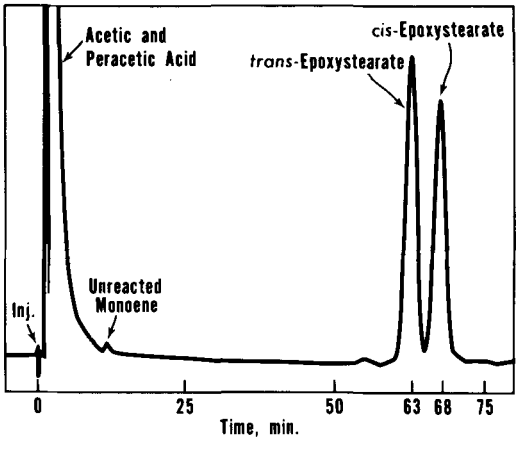

FIG. 1. GC separation of methyl *cis*-9- and *trans*-9-epoxystearate. Operating conditions: Column 10 ft x 1/8 in. 10% EGSS-X, column temperature 200 C, flow rate 38 ml/min helium.

Mixtures of methyl oleate and methyl elaidate were prepared, epoxidized with peracetic acid and analyzed by gas chromatography (GC). Synthetic mixtures containing methyl *trans*-9, *trans*-12-, *cis*-9,*trans*-12-, and *cis*-9,*cis*-12-octadecadienoate were also epoxidized and analyzed by GC.

The epoxidation procedure used for the monoene mixtures consisted of mixing 3 μl of monoene with 150 μl of peracetic acid (2) and allowing the mixture to stand at room temperature for 2 or 3 hr. The diene esters were epoxidized in a similar manner except 300 μl of peracetic acid was used and the reaction time was extended to 4.5-5 hr. Reaction mixtures can be directly analyzed by GC or the mixture can be neutralized with sodium bicarbonate and the epoxidized fatty esters extracted with petroleum ether and then analyzed.

The epoxidized samples were analyzed with a Packard 7400 series GC equipped with flame ionization detectors. Good separation of the methyl *cis*- and *trans*-epoxystearate mixtures was obtained with a 10 ft x 1/8 in. glass column packed with 10% EGSS-X on Chromasorb P. A similar 4 ft x 1/8 in. column was used to separate the diepoxystearate isomers. All analyses were run isothermally at 200 C for the monoenes and at 194 C for the dienes.

A typical GC curve of an epoxidized oleate-elaidate mixture is shown in Figure 1. The *trans*-epoxystearate has the shorter retention time. The percentages of *trans* as determined in three oleate-elaidate mixtures by infrared spectroscopy and GC analysis are compared in Table I.

A sample of isomerized methyl oleate containing both geometric and positional isomers was epoxidized. GC analysis of this sample indicated that the analysis was not affected by the position of thy epoxy group since the GC peaks retained their symmetry and were well

TABLE I

Per Cent Methyl Elaidate

Weight	Infrared[a]	Gas chromatography
11.1	11.7	11.6
51.6	51.2	51.3
77.7	79.9	77.1

[a]AOCS tentative method Cd 14-61.

separated.

The retention times for epoxidized methyl *trans,trans-*, *cis,trans-* and *cis,cis*-9,12-octadecadienoates are in the order listed and are shown in Figure 2 along with the analysis of a synthetic diene mixture. Epoxidized pure *cis, trans*-diene is partially separated into two peaks (Fig. 2-B) and *cis,cis*-diene (Fig. 2-C) is well separated into two peaks.

The two *cis,cis*-diepoxystearate peaks in Figure 2-C (P_1 and P_2) are probably the result of the separation of two diepoxystearate diastereomers (4). These isomers have melting points of 32.4-33 C and 7.5 C and could be expected to have different GC retention times. The *cis,trans*-diepoxystearate diastereomers were separated to a lesser degree and no separation was noted for the *trans,trans*-diepoxystearate diastereomers. The first *cis,cis*-diepoxystearate peak (P_1) overlaps the two partially separated *cis,trans*-diepoxystearate peaks (Fig. 2). Fortunately the ratio of P_1 to P_2 (first and second *cis,cis*-diepoxystearate peaks) can be used to calculate the P_1 integral from the P_2 integral. The P_1 integral can then be subtracted from the combined *cis,trans-* plus P_1 integral and the percentages calculated in the usual manner. Reaction rates for the *cis* isomers were noted to be slightly higher than those for the *trans* isomers. Thus for the most accurate quantitative work, care should be taken to epoxidize at least 95% of the sample before attempting quantitative analysis.

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ACKNOWLEDGMENT

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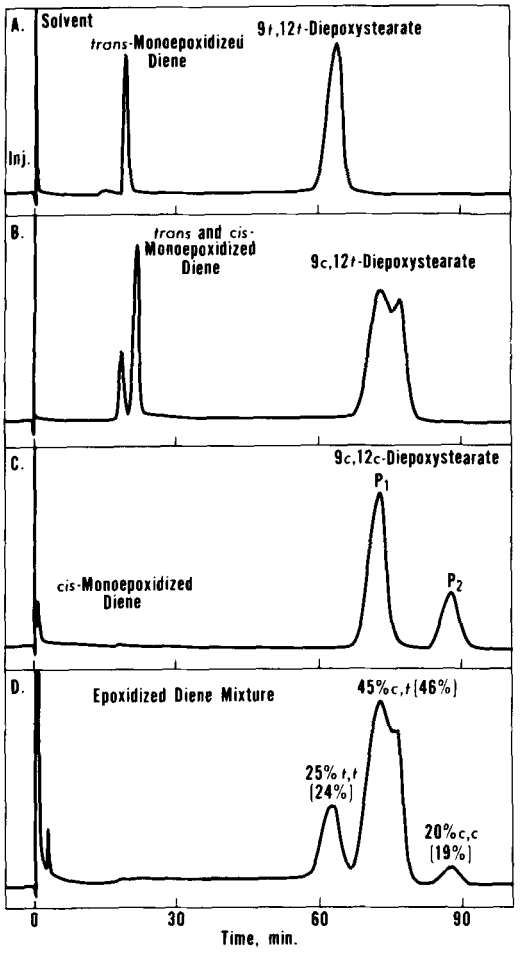

FIG. 2. Retention times for epoxidized methyl 9,12-octadecadienoate geometric isomers and analysis of a synthetic octadecadienoate mixture. In Figure 2-D percentages by GC analysis are given first followed by weight per cent in parentheses. Operating conditions: column 4 ft x 1/8 in. 10% EGSS-X, column temperature 194 C, flow rate 38 ml/min helium.

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[Received April 4, 1971]

Sterols in Five Coastal Spermatophytes

ABSTRACT

Sterolic fractions of *Ruppia maritima* L., *Diplanthera wrightii* Aschers, *Halophila engelmanni* Aschers, *Syringodium filiforme* Kutzing and *Thalassia testudinum* Konig have been isolated. The sterolic fractions were characterized by IR spectroscopy and tentative identities of

separated.

The retention times for epoxidized methyl *trans,trans-*, *cis,trans-* and *cis,cis*-9,12-octadecadienoates are in the order listed and are shown in Figure 2 along with the analysis of a synthetic diene mixture. Epoxidized pure *cis, trans*-diene is partially separated into two peaks (Fig. 2-B) and *cis,cis*-diene (Fig. 2-C) is well separated into two peaks.

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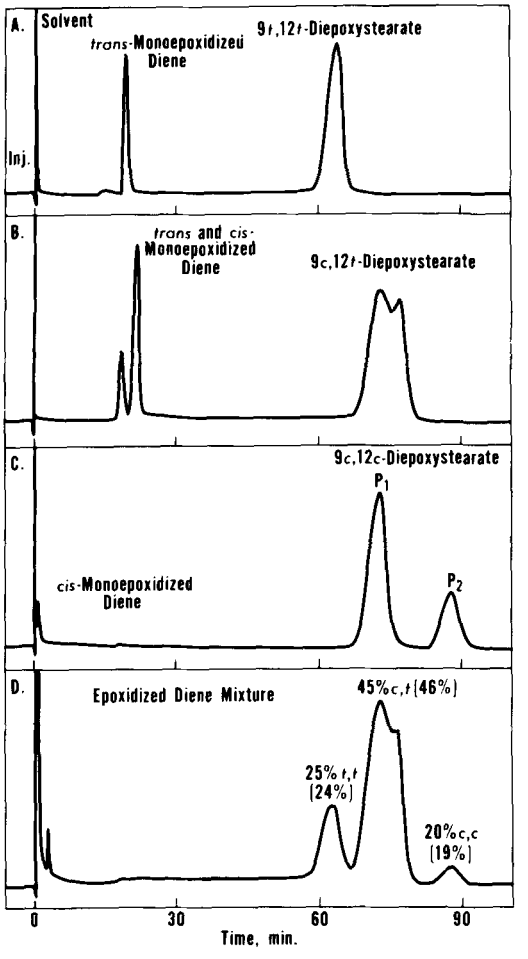

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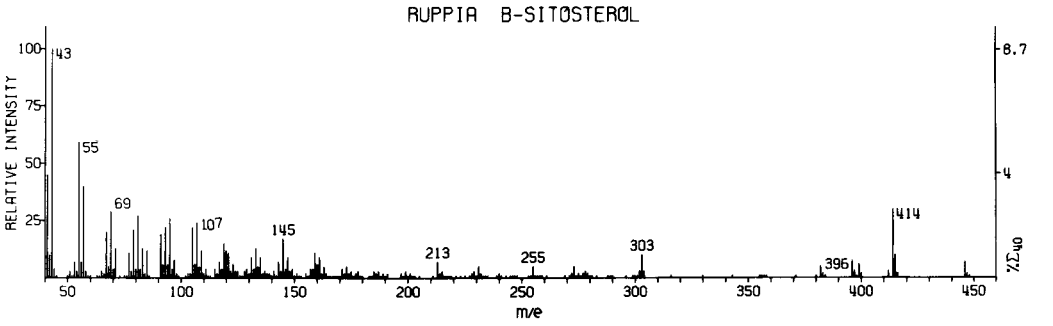

FIG. 1. Mass spectrum of β-sitosterol isolated from *Ruppia maritima*.

their components were determined by gas chromatography and mass spectrometry.

INTRODUCTION

Ruppia maritima L., *Diplanthera wrightii* Aschers, *Halophila engelmanni* Aschers, *Syringodium filiforme* Kutzing (also *Cymodocea manatorum* Aschers) and *Thalassia testudinum* Konig and Sims are spermatophytes which form vast underwater meadows in many areas along the coasts of the Gulf of Mexico (1). Phillips (2) supports the suggestion that these grass-like plants are important in building highly organic lagoonal deposits. Areas in which they occur along the coast of Texas may be of the type which as ancient sediments yield petroleum. We wanted to know what sterols these plants would contribute to sediments, and differences in their sterolic compositions.

MATERIALS AND METHODS

All of the plants except *Ruppia* were collected during October in shallow water of Redfish Bay, Texas. *Ruppia* was collected nearby during the same month in a brackish pond on Mustang Island which is part of the chain of barrier islands along the coast of Texas. Calcareous organisms and other substances were washed from the plants in 1N HCl and tap water. The plants were dried in sunshine or in an oven at 45 C.

Approximately 60 g of each dry plant was extracted with rapidly boiling hexane for 24 hr in 250 ml Soxhlet extractors (this procedure gives 100% recovery of pure cholesterol) and resulting extracts were saponified in refluxing methanolic KOH. Unsaponifiables were extracted from reaction mixtures with ethyl ether and chromatographed on columns of silica gel (Woëlm) in 9 parts redistilled heptane, 1 part ethyl ether. Fractions of 50 ml were collected and those in which the presence of sterols was indicated by thin layer chromatography were combined and weighed.

Thin layer chromatograms on silica gel G were developed in benzene-ethyl acetate (60:20) and visualized after treatment with 5% (by volume) concentrated H_2SO_4 in 95% ethanol and with heat for 15 min. Steroids develop characteristic lavender colors when processed in this manner (3).

Acetyl derivatives were prepared in refluxing acetic anhydride and separated from reaction mixtures by the method of Vishniac (4).

Infrared spectra of the sterolic fractions were measured in KBr discs on a Perkin-Elmer

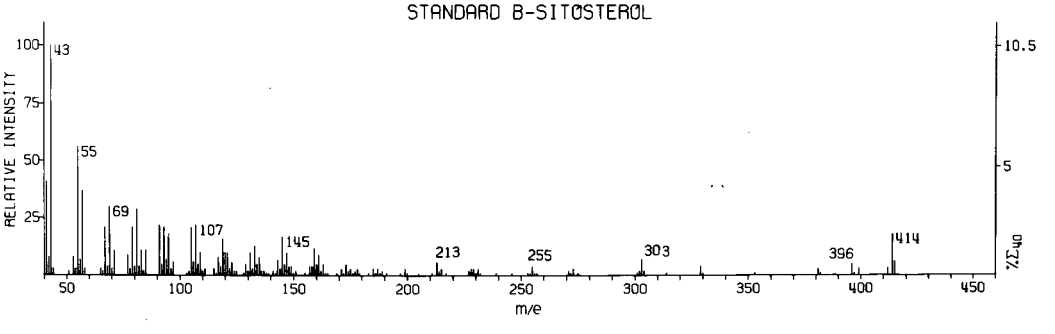

FIG. 2. Mass spectrum of authentic β-sitosterol.

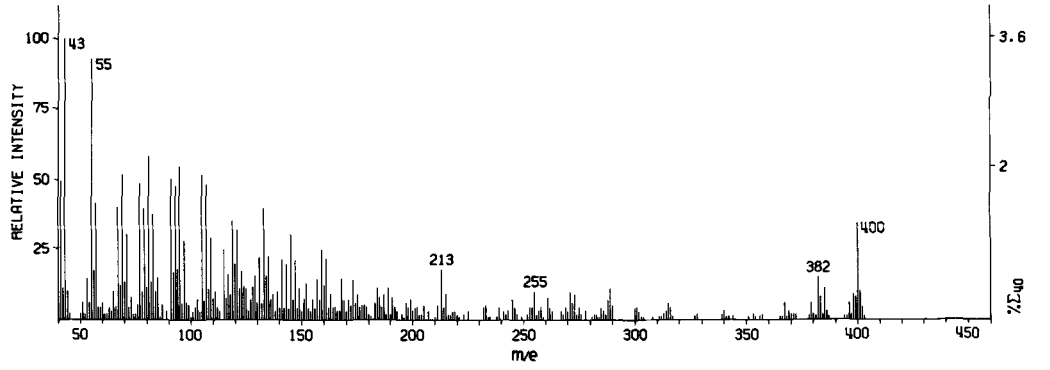

FIG. 3. Mass spectrum of authentic campesterol.

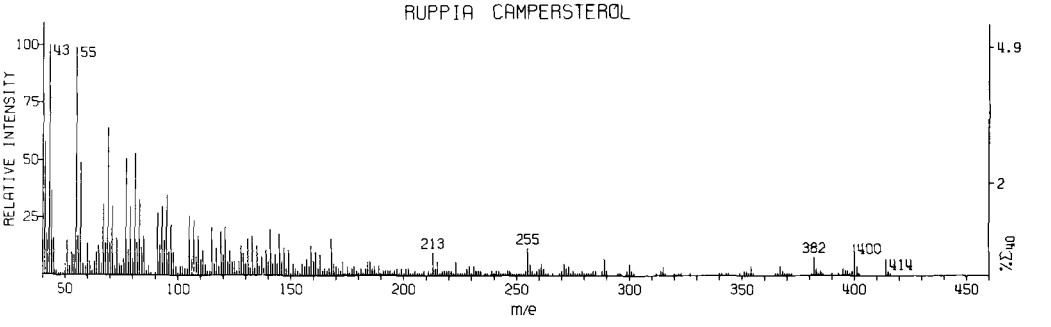

FIG. 4. Mass spectrum of campesterol isolated from *Ruppia maritima*.

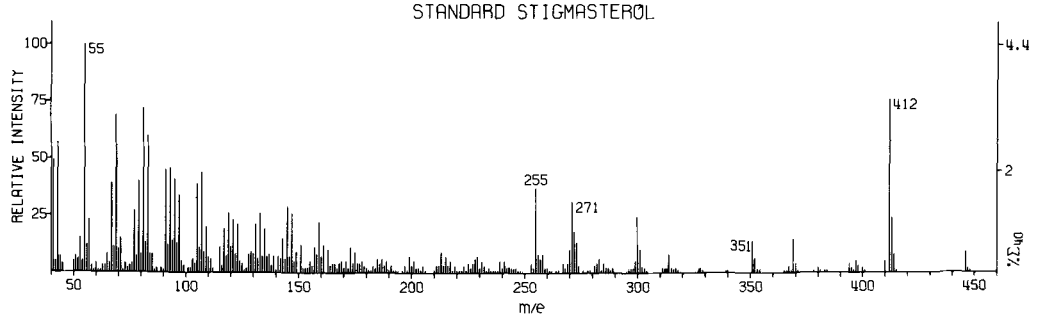

FIG. 5. Mass spectrum of authentic stigmasterol.

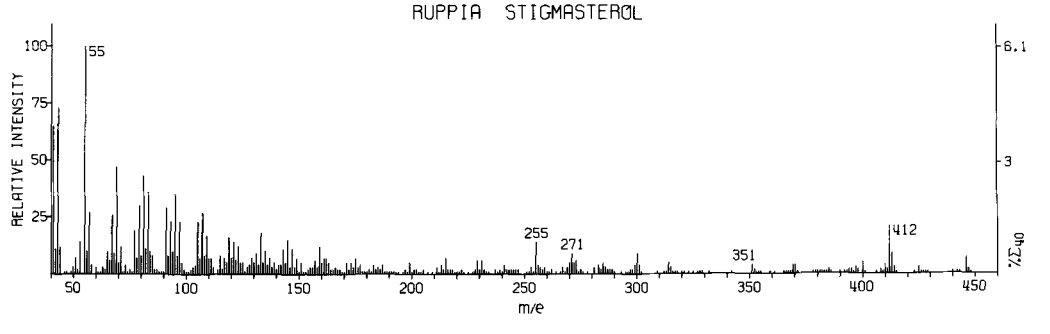

FIG. 6. Mass spectrum of stigmasterol isolated from *Ruppia maritima*.

TABLE I

Lipids of Spermatophytes

Plant	Hexane extract[a]	Unsaponifiables[a]	Sterols[a]	Campesterol[b]	Stigmasterol[b]	β-Sitosterol[b]
Family Ruppiaceae						
Ruppia maritima	2.2	1.1	0.22	22	31	47
Family Zannichelliaceae						
Diplanthera wrightii	1.2	0.20	0.11	10	50	37
Syringodium filiforme	ND[c]	0.47	0.17	2	65	32
Family Hydrocharitaceae						
Halophila engelmanni	1.5	0.22	0.14	13	17	66
Thalassia testudinum	1.1	0.41	0.10	4	25	71

[a]Per cent of dry plant.
[b]Per cent of sterolic fraction.
[c]Not determined.

137B grating spectrophotometer. The fractions were crystallized from 95% ethanol before measurement of their infrared spectra with negligible loss of any component.

Free sterols and steryl acetates were chromatographed in the vapor phase on 6 ft x 1/8 in. columns of 3% JXR on Chromosorb Q in stainless steel tubes. A Perkin-Elmer Model 880 gas chromatograph with dual columns and hydrogen flame detectors was used and the temperature was programmed from 175 to 240 C at 1 C/min. Helium at 50 psi was flowed at 35 ml/min as carrier gas. As they emerged from the columns samples of each component of the sterolic fractions were condensed in glass capillary tubes for later mass spectrometric analysis.

The amount of each component in the sterolic fractions was determined by its corresponding peak area in the gas chromatograms. Peak areas were established by multiplying peak height by peak width at half height.

Mass spectra of individual components were determined in a Consolidated Electrodynamics spectrometer Model 21-110B at 70 ev. Mass spectra of the mixtures were determined at 15 ev.

RESULTS AND DISCUSSION

The IR spectra of the crystalline plant fractions were shown to be compatible with those of authentic sterols; the doublet due to the trans double bond in the side chain of stigmasterol was clear.

Components of each sterolic fraction exhibited gas chromatographic retention times identical to those of campesterol, stigmasterol or β-sitosterol. Coinjection of them with mixtures of those sterolic standards (Applied Science) produced exact overlap of peaks in the gas chromatograms. Comparative gas chromatography in like manner of the acetyl derivatives and of authentic steryl acetates also suggested that each sterolic fraction was composed of campesterol, stigmasterol and β-sitosterol in varying proportions.

Mass spectrometric data supported tentative identifications which were based on gas chromatographic results. Low voltage spectra of the sterolic mixtures from the plants showed for each, molecular ions at m/e 414, m/e 412 and m/e 400 which correspond to β-sitosterol, stigmasterol and campesterol, respectively. Figure 1 shows the mass spectrum of β-sitosterol isolated from *Ruppia*. It compares closely with that of the pure compound shown in Figure 2. The molecular ion is prominent at m/e 414 despite the presence of some "bleed" from the chromatographic column (m/e 446). In the high

mass region one notes the expected loss of a methyl radical and water. Ions at m/e 303, 273, 255 are prominent in the spectrum of β-sitosterol. The ion at m/e 273 in this case represents complete loss of the side chain with a hydrogen rearrangement, m/e 303 partial loss and m/e 255 loss of the 18 mass units of water with the side chain. Figures 3 and 4 show the mass spectra of authentic campesterol and campesterol from *Ruppia*, respectively. The ion at m/e 414 in the spectrum of campesterol from *Ruppia* indicates that β-sitosterol is a contaminant. Figures 5 and 6 show the mass spectra of authentic stigmasterol and stigmasterol from *Ruppia*. In both cases there is good agreement between standard and sample. Mass spectra of corresponding sterols of the other plants also correspond well with the standard spectra. It is noted that mass spectrometric data do not eliminate the possibility of the respective 24-epimers.

Table I shows per cent of each dry plant which is extractable with hexane, amounts of unsaponifiables and sterols in each as well as compositions of their sterolic fractions.

It has been shown that five coastal spermatophytes contain sterols as 0.1% to 0.2% of their dry weights and the sterolic components were tentatively identified as campesterol, stigmasterol and β-sitosterol. In *Halophila engelmanni* and *Thalassia testudinum* (Family Hydrocharitaceae) and *Ruppia maritima* (Family Ruppiaceae) β-sitosterol is indicated as the major sterol; in *Diplanthera wrightii* and *Syringodium filiforme* (Family Zannichelliaceae) stigmasterol predominates. The sterolic fraction of *Ruppia* is peculiar in having almost twice as much campesterol as each of the other corresponding fractions.

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[Revised manuscript received June 16, 1971]

LETTER TO THE EDITOR

Melting Points of Several Enantiomeric Glycerides

Sir: In the preparation of substrates for lipase specificity studies several enantiomeric mono, di and triglycerides have been synthesized (Quinn, J.G., Ph.D. Thesis, University of Connecticut, Storrs, 1967). The melting points of three of these glycerides as well as one specific rotation have not yet been described in the literature. Since this information may be useful to other lipid chemists, these values are herein reported.

These glycerides were synthesized and purified essentially as described by Quinn, J.G., et al., JAOCS 44:439-442, 1967. The monoglyceride, *sn*-glyceryl-3-oleate, mp 22.5-23.5 C, $[\alpha]_D^{22}$ -4.63° (c, 4.0 in pyridine) was prepared from 1,2-isopropylidene *sn*-glycerol (Baer, E., "Biochemical Preparations," Vol. 2, Edited by E.G. Ball, John Wiley and Sons, Inc., 1952, p. 31-38) and oleoyl chloride.

Diglyceride was prepared by acylating *sn*-glyceryl-3-palmitate with oleoyl chloride (*sn*-glyceryl-1-oleate-3-palmitate, mp 48.5-48.9 C). The triglyceride, *sn*-glyceryl-1-2-dioleate-3-palmitate, mp 18.5-19.0 C, was isolated as a by-product from the synthesis of the above diglyceride.

The purity of the glycerides was determined by thin layer chromatography and their fatty acid composition by gas liquid chromatography (Quinn, J.G., Ph.D. Thesis, 1967). A stereospecific analysis (Sampugna, J., and R.G. Jensen, Lipids 3:519-529, 1968) was used to characterize the diglyceride and the triglyceride subjected to pancreatic lipolysis (Jensen, et al., Lipids 5:580-581, 1970). On the basis of the methods employed, the purity and correctness of position is estimated to approach 99%.

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ERRATUM

Page numbers were omitted from the following references in the August issue of LIPIDS:

P. 530, 25. de la Roche, I.A., E.J. Weber and D.E. Alexander, Ibid. 6:537-540 (1971).

P. 536, 1. Weber, E.J., I.A. de la Roche and D.E. Alexander, Lipids 6:525-530 (1971).
2. de la Roche, I.A., E.J. Weber and D.E. Alexander, Ibid. 6:537-540 (1971).

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Lipids and the Pill

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ABSTRACT

The widespread use of anovulatory compounds and the well-known effects of sex hormones on various aspects of metabolism prompted this review of our work and the work of others on observed changes in lipid metabolism resulting from the administration of oral contraceptives and their components. In the rat, female sex hormone administration results in a decreased plasma cholesterol level, an accumulation of cholesterol in liver and a decreased hepatic cholesterol biosynthesis. On the other hand, cholesterol biosynthesis is enhanced in ovaries and adrenals. There is also a diminished alpha lipoprotein content and a corresponding decrease in the alpha/beta lipoprotein ratio. In some cases these changes are comparable to those observed during pregnancy. The results of sex hormone administration to women are more variable. In this case the most often observed effect is hypertriglyceridemia. Changes in lipoprotein content and distribution are also evident and may be the result of changes in metabolism in the liver, e.g., lipid synthesis or lipid transport from liver to plasma and tissues, or both. Many of these changes may be mediated indirectly through the action of estrogen-progestin on other hormones. In both species the effects of oral contraceptives are attributable principally to the estrogen component. The combination of estrogen with progestin compounds, which constitutes the oral contraceptive, modifies the effects of estrogen administered alone.

INTRODUCTION

The popularity of "the pill" is a remarkable event of modern scientific technology and excellent public relations. One decade following its introduction, approximately 20 million women throughout the world are using the various estrogen-progesterone mixtures available as anti-ovulatory drugs, not for therapy but to intentionally modify certain natural processes relating to reproduction. Public health and medical authorities have viewed this phenomenon with mixed feelings. On the one hand is the great promise offered in the control of threatened overpopulation; the other consideration is the potential danger of undesirable and possibly still unknown accompanying symptoms. In spite of continuing reports of unpleasant and even dangerous side effects, the relative ease of use of anovulatory compounds and their effectiveness in preventing pregnancies have promoted their widespread acceptance.

Oral contraceptives are mixtures of hormones or hormone-like synthetic compounds, or both, which influence a variety of metabolic processes and which induce biochemical changes over and above those necessary for conception. The effects of oral contraceptives on various aspects of intermediary metabolism have been reviewed by many investigators. This review will be limited to effects on lipid metabolism.

HISTORICAL BACKGROUND

Approximately 30 years ago it was reported (1) that the administration of estrogens to women inhibited ovulation. At about the same time Makepeace et al. (2) demonstrated that progesterone had similar properties. Subsequently the anovulatory activity of a series of synthetic progestins was reported by Pincus and Chang (3) and others (4) and as a result of these investigations, research toward the development of safe, orally active steroid compounds was greatly stimulated. Commercial products to control ovulation became available shortly thereafter.

Clinical trials of these newly developed products were performed in Puerto Rico (5,6) and as a result, in 1957, the first of these compounds, Enovid, was released, first as treatment for menstrual disorders and then later, in 1960, as an oral contraceptive. The combination in this drug of the orally active 19-nor-progestational steroid together with a small amount of estrogen resulted in an inhibition of ovulation, although the regular, cyclic menstrual period was maintained. The original dose level was a mixture of 10 mg norethynodrel and 0.15 mg of ethinyl estradiol-3-methyl ether (mestranol); these levels have since been reduced. Subsequently, in addition to 19-nor-steroids, other groups of compounds were found to be effective in this regard, e.g., the

17-acetoxy-progesterone and its derivatives (7). The 19-nor-progestational steroids, e.g., norethynodrel, can undergo conversion into biologically active estrogens, thus potentiating the effects of the estrogen originally present in the combined drug. Rat liver preparations have been shown to convert norethynodrel to the 3-alpha and 3-beta diols by means of dehydrogenases and to carry out aromatization to form the estrogenic compound 17-alpha ethinyl estradiol (8). A variety of compounds are available at present using the minimal effective dose of estrogen, since most of metabolic effects are apparently due principally to the estrogen component of the drug.

MODE OF ACTION OF ANOVULATORY DRUGS

The primary action of the combined oral contraceptives is a suppression of the synthesis or of the secretion of pituitary gonadotropins (9), or both. The estrogenic component of the anovulatory drug is chiefly responsible for the suppression of the secretion of the follicle-stimulating hormone of the pituitary resulting in incomplete follicular maturation and interference with ovulation. Also changes in cervical mucus render it hostile to the penetration by sperm. In addition progestin can prevent the secretion of the luteinizing hormone (the "LH peak") necessary to trigger ovulation (10,11). Other investigators feel that the estrogen component is chiefly responsible for the inhibition of ovulation with progestin lending constancy to the cycle (12).

METABOLIC EFFECTS OF ANOVULATORY DRUGS UNRELATED TO LIPIDS

To date a plethora of supposedly unrelated metabolic changes has been reported as resulting from oral contraceptive administration, e.g., a disturbance in tryptophan metabolism suggestive of vitamin B_6 depletion, an increased circulating level of blood coagulation factors, an elevated renin-substrate level and increased aldosterone secretion, an increased circulating level of serum iron, copper, cortisol, thyroxine, insulin, growth hormone, serum transaminase and an increased retention of bromsulphthalein, an altered carbohydrate metabolism (13), a decreased serum and urine magnesium (14), lowered plasma zinc levels (15), and an altered platelet behavior similar to that observed in atherosclerosis (16).

EFFECTS OF THE FEMALE SEX HORMONES ON LIPID METABOLISM

Animal Studies

Effects on Cholesterol Content of Liver and Plasma. Oral contraceptives are synthetic steroid mixtures with biological activity simulating that of female sex hormones. Sex hormones have been shown to affect various phases of lipid metabolism. There have been numerous reports in the literature indicating sex differences in lipid metabolism of the rat. In general female animals have higher plasma cholesterol but lower liver cholesterol levels than do males (17). Females also have higher rates of hepatic cholesterol synthesis (18) which are decreased after gonadectomy (19-21), although in castrated female rats Kritchevsky et al. (22) have reported no significant differences in this regard between control and experimental animals.

Estradiol benzoate administration to male rats has resulted in a marked decrease in plasma total lipid concentration (23) as well as in a lowered plasma cholesterol concentration, and higher liver cholesterol levels. Boyd (24) also reported a decreased plasma cholesterol level in male rats following the administration of a variety of estrogens. Similarly Premarin (a mixture of equilin, estrone sulfate and estriol glucuronide), fed to hyperlipemic rats at two levels (8 or 12 mg/kg) was equally effective in reducing serum cholesterol, triglycerides and atherosclerotic lesions. It was also observed that the higher dosage increased the corticosterone level in plasma and decreased serum albumin level (25). Merola et al. (26) explained these changes by suggesting that the primary effect of estrogens is a redistribution of the cholesterol between the plasma and the liver. They observed that the oral administration of estrogens to male rats caused a decrease in serum cholesterol levels, and even though hepatic cholesterol synthesis was inhibited in slices and homogenates, no such response could be elicited in in vivo studies. Morin (27) also suggested the possibility that estradiol may effect a redistribution of cholesterol by concentrating it in the hepatic pool. He observed that estrogen-treated rats showed an increased hepatic concentration of cholesterol; however at the same time an increased degradation and excretion of cholesterol was reported.

Fatty Acid Composition of Phospholipids and Cholesteryl Esters. In vitro studies reported by Schweppe and Jungmann (28) showed that the action of 17-beta-estradiol on cholesterol esterification by rat liver microsomes depended on the concentration of hormone used. Cholesterol oleate formation increased inversely with hormone concentration, being the highest at the lowest levels of hormone, whereas the esterification of cholesterol with palmitate and linoleate responded in an irregular fashion to changes in hormone level. These authors believe

TABLE I

Composition of Some of the Oral Contraceptives[a]

Trade name combination	Progestin component	Estrogen component	Ratio prog.-estr.
Anovlar	Norethindrone acetate, 4 mg	Ethinyl estradiol, 0.05 mg	80:1
Enovid	Norethynodrel, 9.85 mg	Mestranol, 0.15 mg	66:1
Enovid E	Norethynodrel, 2.5 mg	Mestranol, 0.1 mg	25:1
Norinyl	Norethindrone, 2 mg	Mestranol, 0.1 mg	20:1
Norlestrin	Norethindrone acetate, 2.5 mg	Ethinyl estradiol, 0.05 mg	50:1
Ortho-Novum 10	Norethindrone, 10 mg	Mestranol, 0.06 mg	167:1
Ortho-Novum 1	Norethindrone, 1 mg	Mestranol, 0.05 mg	20:1
Ovulen	Ethynodiol diacetate, 1 mg	Mestranol, 0.1 mg	10:1
Provest	Medroxyprogesterone acetate, 10 mg	Ethinyl estradiol, 0.05 mg	200:1
Volidan	Megestrol, 4 mg	Ethinyl estradiol, 0.05 mg	80:1
Sequential			
C-Quens	Chlormadinone acetate, 2 mg	Mestranol, 0.08 mg	
Ortho Novum	Norethindrone, 2 mg	Mestranol, 0.08 mg	

[a]Adapted from S.M. Kalman Ann. Rev. Pharmacol. 9:363 (1969).

that estrogenic hormones exert effects on cholesterol metabolism at three levels: (a) on the biosynthesis of cholesterol from acetate or mevalonate; (b) on the esterification of cholesterol; (c) on the lipoprotein transport complex. In the rat 17-beta-estradiol was found to reduce in vitro hepatic lipogenesis by 50% (particularly the synthesis of triglycerides and phospholipids) while cholesterol synthesis was not seriously affected. There was a marked increase in the polyunsaturated fatty acid content of triglycerides accompanied by a decrease in concentration of saturated and monoenoic fatty acids (29,30). On the other hand estrone, estriol and estradiol in small amounts stimulated lipogenesis in adipose tissue of female rats in vitro. These hormones acted synergistically with insulin (31).

The activity of lecithin-cholesterol acyl transferase (LCAT) in rat plasma as reflected by the extent of esterification of free cholesterol was found to be higher in mature female rats than in the males (32). These authors suggested that fatty acid composition of plasma lipid fractions was sex-dependent; there was a higher concentration of unsaturated fatty acids in the plasma lipids of the female. Lyman et al. (33) have also shown that female rats had higher amounts of serum polyunsaturated fatty acids; plasma lecithins of female rats contained more arachidonate than did plasma lecithins of male rats. Subsequently these investigators (34) reported that during the development of EFA deficiency female rats and estrogen-treated, castrated male rats maintained higher proportions of arachidonate in plasma phospholipids and cholesteryl esters than did intact males. Similar results showing the sparing of polyunsaturation in fatty acids by estrogens have been previously reported by Aftergood and Alfin-Slater (20).

On the other hand when other phases of lipid metabolism were studied Christiansen et al. (35) found no sex differences in the ability of rats to perform dehydrogenation and chain elongation reactions during which linoleate was converted to arachidonate. In their in vitro studies these authors observed that female rats were able to effect elongation of palmitic to stearic acid with a capacity twice as high as did the males. However the incorporation of arachidonate into complex lipids was not influenced by the sex of the animal.

Lipoprotein Composition. The composition of serum lipoproteins is affected by estrogen administration. When Hill and Dvornik (36) compared normal male rat serum lipoproteins with those from rats previously treated with 17-beta-estradiol, they observed after treatment an elevation of triglyceride in the very low density lipoprotein (VLDL) fraction as well as a decrease in the cholesterol content in the same fraction. Phospholipids were elevated in both the VLDL and high density lipoprotein (HDL) fractions.

Bile Production and Liver Function. Cholesterol catabolism has also been shown to be sex-linked. Mitochondria from intact female rats were found to oxidize cholesterol to a greater extent than mitochondria from intact males (37). Saini and Patrick (38) reported that the incorporation of radioactivity from labeled cholesterol was increased in bile of rat liver perfused with estrone, although there was no change in the rate of bile acid formation. Treatment with estradiol was shown to increase chenodeoxycholic acid production in rats (39) while bile cholesterol content was diminished.

On the other hand Kreek et al. (40) observed that in estrogen-treated rats, bile flow was reduced to 50% that of controls. This estrogen-induced cholestasis may involve an enhanced diffusion of materials from bile to blood as well as an inhibition of active transport in the opposite direction (41). The impairment of liver function due to estrogens is reflected by an increase in the bromsulphthalein (BSP) retention time (42). Similar disturbances in liver function also occur during pregnancy (43,44).

Ovarian and Adrenal Cholesterol Relationships. Since steroid hormone production in the ovary is closely regulated by pituitary hormones and since cholesterol is the precursor of steroid hormones, it is not surprising to find changes in ovarian cholesterol levels as a result of sex hormone administration. One of the most dramatic effects of luteinizing hormone upon the ovary is its ability to deplete cholesterol stores; the depletion coincides with the time of increased progestin secretion, and evidence has been provided that a precursor-product relationship exists between these compounds. LH alone causes estrogen production by *corpora lutea*, while in the interstitial tissue, LH requires other factors for estrogen production. LH causes an accumulation of cholesterol by interstitial tissue but not by the luteal tissue (45). Herbst (46) also found a depletion of cholesterol ester in the rat ovary in response to LH. Esterified cholesterol was found to be the substrate for the in vitro synthesis (47) as well as the in vivo synthesis (48) of progesterone by luteinized ovarian slices. Behrman and Armstrong (49) have observed that an iv administration of LH to rats results in a significant increase in cholesterol hydrolase activity in the ovaries. It is possible that the intracellular transfer of stored (esterified) cholesterol into mitochondria for conversion into pregnenolone is stimulated by this hormone. Cholesterol ester depletion occurs simultaneously with steroid secretion in both adrenal and ovarian tissue. Increased ester hydrolysis may be the cause rather than the effect of an increased cholesterol-pregnenolone conversion. LH exerts an inhibitory action on cholesterol esterification in the ovary.

There is a circadian rhythm in the ovarian cholesterol content (50). A 50% decrease in cholesterol content takes place on the day of proestrus. Depletion occurs during metaestrus and diestrus as well. Similarly Hunter and Stewart (51) observed a decrease in plasma free cholesterol between proestrus and diestrus and a decrease in plasma esterified cholesterol between estrus and diestrus. However no such rhythm is evident when there is constant exposure to light.

Some variations in ovarian cholesterol levels due to strain differences have been observed. A very thorough review of ovarian metabolism and steroid biosynthesis has been recently published by Armstrong (52).

There is considerable evidence that gonadal hormones exert an important influence on adrenocortical function. Estrogens lead to increased plasma corticosteroid levels, enlarged adrenal size, enhanced response of adrenal steroid production of ACTH, and also increased synthesis of ACTH by the pituitary (53). Even the intermediate biochemical reactions in the adrenal have been found to be influenced by sex hormones, i.e., it has been reported recently that estradiol stimulates the hydroxylation of desoxycorticosterone (54). It appears that the pituitary is particularly responsible for the hypocholesterolemic effect of estrogen in rats since no such effects are present in immature or hypophysectomized aminals (55,56). The existence of a hypophyseal factor involved in the regulation of cholesterol metabolism and which is subject to stimulation by estrogen has been postulated.

It has been suggested (57) that large doses of estrogen inhibit steroid secretion whereas smaller doses are stimulatory. Uchida et al. (58) showed this biphasic effect of estrogen on rat plasma cholesterol levels. Apparently a hypercholesterolemic effect is produced slowly by a small dose of estrogen administered over a long period of time, whereas a hypocholesterolemic effect is caused rapidly by a higher dose of estrogen administered for a short time period. In addition there is a difference between the effects produced by different types of estrogenic compounds. For instance estradiol, estrone, estriol and ethinyl estradiol produce a transient "hypo" effect followed by a marked "hyper" effect, whereas hexestrol, diethyl stilbestrol and norethynodrel produce only a transient "hypo" effect.

Recapitulation. There is no doubt that female sex hormones exert a regulatory role in lipid metabolism of the rat. Conflicting results appearing in some investigations are probably due to a variety of experimental factors, e.g., the type of estrogen preparation used, the dose level and its method of application, the length of exposure of the animal to the estrogen compound, the age of the animal, the in vivo vs. in vitro conditions and techniques employed, and the nutritional status of the animal at the time of the accompanying metabolic stress. When large doses of estrogen preparations are given to the normal adult female rat certain

events become apparent, i.e., plasma cholesterol is decreased and hepatic cholesterol synthesis is inhibited. This may result from either an estrogen-induced redistribution of lipids, cholesterol in particular, in the organism, or from a selective catabolism of these lipids. Liver function is also affected. Lipid distribution among various lipoproteins may be changed. The metabolism of steroids in ovaries and adrenals is greatly stimulated.

A comparison between the effect of the administration of female sex hormones and the pregnancy state on lipid metabolism reveals some similarities. Apparently fluctuations in plasma cholesterol levels have been observed in the pregnant rat, with the free cholesterol component increasing during the second half of gestation (51); on the other hand a decline in total cholesterol in the pregnant monkey was reported within one month following conception (59).

Human Studies

Studies on the effect of female sex hormones on lipid metabolism were stimulated due to the observations that there were sex differences in susceptibility to the disease atherosclerosis; pre-menopausal women were found to be more resistant to the disease than were men in comparable age brackets. As a result female sex hormones were introduced as therapy to male patients suffering from atherosclerotic involvements and a better understanding evolved of the role of estrogens in lipid metabolism mediated through investigations on blood lipids.

Serum Lipid Levels. Plasma cholesterol levels are related to age, diet and sex. All plasma lipid fractions have been found to be lower in menstruating, 50-year-old women than in postmenopausal females of the same age, due presumably to the hormonal effect (60). During the menstrual cycle many blood constituents vary considerably, probably also a function of variations in hormonal activity. In general although "femaleness" may be responsible for the lower serum cholesterol level found in women, this sex difference is not always consistently seen, particularly in populations other than the U.S. Evidently it is being attenuated by other environmental factors (61).

Some of the results on the effects of estrogens on lipid metabolism obtained by investigators seem to be controversial. Therefore although estradiol was found to be instrumental in reducing lysolecithin levels (62), the same authors (63), in a study of 25 women, reported no significant differences in levels of cholesterol, phospholipids or EFA during the 8th through 23rd day of the menstrual cycle. On the other hand Hunter and Stewart (51) reported increasing plasma cholesterol levels between menstruation and ovulation. Free cholesterol was significantly reduced following ovulation. Estrogen administration was reported to diminish the cholesterol-phospholipid ratio by increasing serum phospholipid concentration or lowering serum cholesterol, or both (64), and to increase serum triglyceride concentration (65). An increased incorporation of labeled acetate into lecithin of human peripheral arteries in vitro due to estradiol was reported by Morin (66), while Hagopian and Robinson (67) have observed an increase in lecithin in postmenopausal women treated with estrogen for a month.

Liver Function. An increase in bromsulphthalein retention has been reported in 5 of 9 women treated with estrogen (68).

Lipoprotein Distribution. The three major groups of lipoproteins characterized by flotation in the ultracentrifuge and by migration in various electrophoretic media are alpha lipoproteins, or HD fraction, which carry some cholesterol and most of the plasma phospholipid; beta-lipoproteins, or LD, which contain most of the cholesterol and the triglyceride rich, very low density (VLD), pre-beta, or alpha$_2$ migrating lipoprotein fraction (69).

Estrogens are apparently responsible for cholesterol distribution between the lipoprotein fractions. An increase in the cholesterol content of HDL and a decrease in LDL cholesterol during estrogen administration were first observed by Barr et al. (70). Progestins have little or no effect on these lipoproteins (71). The effect of estrogen appears to be both on the number of circulating lipoprotein aggregates and on the chemical composition of each lipoprotein class. According to Furman et al. (67,72) estrogen administration increases the lipid content of the HD and the VLD lipoproteins resulting in higher serum phospholipid and triglycerides and lower cholesterol: phospholipid ratios. The cholesterol response varies depending on magnitude and direction of change in the cholesterol of beta lipoproteins. The increased amounts of cholesterol and phospholipids in the HD lipoproteins suggest increased levels of this lipoprotein fraction. The increase in lipid-protein ratio of VLD lipoprotein is due primarily to the increase in triglycerides. The same investigator (73) later suggested that estrogen should be regarded as a cholesterol lowering hormone in respect to the cholesterol content of the HDL particle although it increases the concentration of HDL particles in the blood.

Lipoprotein Lipase. Hypertriglyceridemia induced by corticoid treatment has been found to be associated with a decreased postheparin lipolytic activity (74). It has been suggested that since this enzyme is critical for the assimilation of circulating lipoprotein triglycerides, its deficiency could well lead to hypertriglyceridemia. Hormones other than cortisone compounds, e.g., insulin, also affect this post-heparin lipoprotein lipase resulting in high triglyceride levels in blood (75). Hypertriglyceridemia in pregnancy in animals has been associated also with low lipoprotein lipase (76). This enzyme is released into the circulation by the intravenous administration of heparin. Fabian et al. (77) have shown that post-heparin lipolytic activity was decreased after estrogen administration.

Pregnancy. During pregnancy the organism is exposed to high estrogen levels. As a result lipoprotein lipase levels are decreased (78), levels of FFA are higher than in nonpregnant women, and there is a decreased utilization of fatty acids in tissues. During the 15-18th week of pregnancy serum cholesterol, phospholipid and triglyceride concentrations begin to increase in conjunction with increased placental estrogen synthesis, as is evidenced by urinary estriol excretion (79). The decline of these lipids begins in the first postpartum week (80). In an extensive study of fatty acid composition of serum lipids during pregnancy, deAlvarez et al. (81) have shown that not only do the serum FFA increase progressively but a trend toward saturation of the fatty acids, manifested by a reduction in linoleic acid, occurs in middle to late pregnancy. The authors postulate a tentative mechanism of fatty acid metabolism in pregnancy as follows. Under stress, e.g., pregnancy, growth hormone is released from the anterior pituitary and through its diabetogenic action decreases carbohydrate utilization while enhancing fat mobilization. Growth hormone together with corticosteroids stimulate a major lipoprotein production and antagonize insulin action. As a result there is decreased carbohydrate tolerance associated with hyperlipemia during pregnancy, an increased deposit of fat and the appearance of gestational diabetes during pregnancy.

Recapitulation. Studies of the effects of sex hormones on lipid metabolism in humans yield more variable results than those which are obtained after investigations on rats. The response of serum cholesterol to estrogen administration is not always definitive; some variations in cholesterol content occur during the menstrual cycle and a general lipemia is observed during pregnancy. However hypertriglyceridemia is a characteristic response to estrogen therapy. An effect on liver function involving biosynthesis or release of certain lipid components, or both, may be a partial cause of observed changes in lipoprotein distribution and content. At the same time the activity of lipoprotein lipase is under hormonal control and it, in turn, may be involved in establishing levels of lipoproteins in general and of individual lipids in particular.

ANOVULATORY DRUGS AND THEIR EFFECT ON LIPID METABOLISM

The available oral contraceptives are of two types: the progestin-estrogen combinations which are given throughout each cycle of treatment, and the sequential preparations consisting of estrogen administration for the first five days. The use of the first type of the drug is much more widespread, perhaps as a result of its more frequent use in animal investigations. There are dozens of the combination type products available; these differ mainly in the nature and quantity of the progestin component (82). The composition of some of the most often used oral contraceptives is presented in Table I. As the estrogen component only ethinyl estradiol or mestranol (ethinyl estradiol-3-methyl ether) are used. The way in which these preparations are administered may be of importance. For example medroxyprogesterone acetate given orally exerts no inhibitory effect on the secretion of gonadotropins; however when injected it exhibits an intense and persistent pituitary-inhibitory action (83). Although the estrogen component seems to be the more important ingredient of the combination, the two components may act synergistically (84).

In order to minimize the metabolic side effects of oral contraceptives which often accompany their desired anovulatory activity, as a result of animal experimentation, the effective dose levels have been decreased over the years.

Animal Studies

There are few reports available which involve the use of oral contraceptives in rats. It has been tentatively established that the daily dose required to prevent conception in the rat is 200 μg of progestin per kilogram of body weight (85). This is comparable to the level of 50 μg Enovid (48 μg progestin; 2 μg estrogen) per rat (86) and the levels of 191 μg norethynodrel (progestin) and 2.8 μg mestranol (estrogen) per kilogram of body weight per day administered by Yang et al. (87), and 10 μg of estrogen with 100 μg progestin per rat per day administered by Banik et al. (88).

Tissue Cholesterol Levels, Cholesterol Biosynthesis and Excretion. Extensive studies of the effect of an oral contraceptive drug, Enovid E, have been performed by Aftergood et al. (89). These authors originally used a high, unphysiological dose of the drug to test the toxicity of the preparation and to exaggerate the appearance of metabolic effects. Thus in the early studies 1 mg of Enovid E (human dose is 2.6 mg/day, which consists of 2.5 mg norethynodrel and 0.1 mg mestranol) was administered to adult female rats for seven days. In later studies both the length of the experimental period and the dose level have been decreased. The results obtained were similar regardless of dose level (90,91).

In general Enovid E administration resulted in a marked decrease in the level of total cholesterol in plasma and adrenals. The decrease in adrenal cholesterol was manifested primarily by decreases in the esterified cholesterol fraction. Cholesterol in the liver was substantially elevated. Similar trends have been previously obtained when castrated female rats were treated with estradiol benzoate (20). In plasma cholesterol esters a decrease in arachidonic acid content was attributed to Enovid administration. In adrenals and ovaries the decreased total cholesterol content was reflected by the decreased content of arachidonic and docosatetraenoic acids (characteristic fatty acids esterified with cholesterol in these organs). Previously an increased hepatic cholesterol had been observed in castrated female rats given estradiol benzoate, at an approximate level of 0.17 mg hormone per rat per day. When cholesterol biosynthesis from 1-^{14}C-acetate was examined in vitro studies it was found that sterol synthesis was depressed in livers of Enovid-treated animals whereas it was significantly enhanced in ovaries and adrenals, indicating perhaps an increased requirement for cholesterol in those organs which are the main sites for steroid hormone synthesis. At the same time less cholesterol was excreted in the feces. A return to normal conditions was rapidly achieved after treatment was discontinued; however repeated dosing yielded values comparable to the initial response (92); no tolerance or intolerance to the drug seems to have been established. The administration of low physiological doses of Enovid E (0.052 mg/day/rat) resulted in findings similar to those obtained when the dose level was 20 times higher. Esterified cholesterol decreased rapidly in the serum and in adrenals and accumulated in the liver. Serum phospholipids also decreased. Alpha-lipoproteins, ordinarily quite abundant in the rat, were significantly decreased as was shown by the decrease in alpha-beta lipoprotein ratios. These effects were shown not to be secondary to a decreased food consumption (91).

Comparisons Between Various Anovulatory Drugs. A comparative study of the effects of two anovulatory drugs, namely, Enovid E and Provest (a mixture of medroxyprogesterone acetate and ethinyl estradiol) were also performed (90). Both of these drugs are effective anovulatory agents and are comparable in their activity in this respect. However at comparable dose levels administered to rats, their effects on cholesterol metabolism vary in intensity with Enovid exerting a much greater influence on all of the variables examined. The reasons for these differences may be due to the amount and kind of progestin and/or estrogen component and/or the ratio of progestin to estrogen within the product. Vyas (93). has reported changes occurring in rat tissue cholesterol levels following an oral or intraperitoneal administration of 0.1 mg Anovlar (norethindrone acetate, 4 mg + ethinyl estradiol, 0.05 mg per kilogram of the rat for 175 days. In general increases of hepatic cholesterol were accompanied by decreases in cholesterol of serum, adrenals and ovaries. The author suggests that some of these effects may be due to the formation of estrogenic metabolites.

Comparison of the Effects of the Components of Anovulatory Drugs. The effects of the separate administration of the components of one of these contraceptive drugs (Provest) were compared with the effects of the combined product (94). The dose used, 80% of the human dose, is unphysiological for the rat but was used to magnify the effects previously observed. As expected the mixture of the progestin and estrogen compounds produced the same response as did the original, commercially prepared product. On the other hand progestin when administered alone was without effect on rat lipids whereas the estrogen component alone yielded enhanced results. The combination of the two drugs modified the enhanced effect produced by estrogens alone. This was true as far as effects on liver, serum and adrenal cholesterol levels, as well as on serum arachidonate and adrenal docosatetraenoate, were concerned.

Recapitulation. In general in the rat the effects of oral contraceptives simulate those of estrogenic sex hormones. However the combination of estrogen-progestin compounds modifies the effects of estrogen administered alone.

Human Studies

The effects of oral contraceptives on human

blood lipids have attracted wide attention due to their possible influence on the etiology and development of vascular disease, especially thrombogenesis. Extensive, long term effects of observed changes in short term experiments are not available at present, although reports dealing with the various areas under investigation are now accumulating.

Liver Function. Since the main organ concerned with lipid metabolism is the liver, it is pertinent to point out that mild hepatic damage, similar to cholestasis of pregnancy, has been reported in many women using oral contraceptive drugs (95). This author claims however that most of the adverse reactions thought to be associated with the oral contraceptives occur spontaneously in the population, albeit as a very low rate. Apparently treatment with oral contraceptives puts an added load on the metabolic functions of the liver. An increased serum transaminase and alkaline phosphatase are also common in anovulatory drug-treated patients (96). Some changes in bile canaliculi and liver cell mitochondria, of the same type as have been seen in jaundiced patients, have also been observed (97). Moderate dilatation and vesiculation of endoplasmic reticulum, as well as fatty vacuolations of the liver cells, have been reported by Martinez-Manautou et al. (98) in women undergoing contraceptive therapy. Also striking alterations in plasma proteins, particularly those known to be synthesized by the liver, have been observed in women taking oral contraceptives (99). The effects of oral contraceptives paralleled those produced by late pregnancy. Similarly Burton (100) reports that urinary total coproporphyrin (bile pigment) concentration was increased in 26% of women taking oral contraceptives while 16% of women had mild hepatic dysfunction. According to Arias (101) oral contraceptive agents have resulted in a reduced hepatic excretory function in every patient studied. There is evidence for both a primary impairment of bile salt excretion as well as an enhancement of the back diffusion of substance from bile to plasma (42). According to Adlercreutz and Tenhunen (102) female sex hormones affect the liver cell in all the subjects but only some react with cholestasis, possibly due to a defect of the drug metabolizing system. It is possible that steroids interfere with the secretion of the conjugated cholates essential for micelle formation. While estrogen would affect mainly protein synthesis in the rough endoplasmic reticulum, progestin might affect the smooth ER and the drug metabolizing system.

Lipoprotein Distribution and Plasma Lipid Levels. The fact that the liver excretory system is affected as shown by disturbances in bile metabolism and in some of its enzymes may be related to changes observed in lipoprotein concentrations. The effect on lipoprotein synthesis would be localized in the liver.

The effects of contraceptive agents on lipoproteins, plasma cholesterol and TG levels have produced conflicting reports. In 1966 Wynn et al. (103) reported that LDL and VLDL levels were markedly increased in women taking oral contraceptives whereas total cholesterol was only slightly increased. The authors suspected that these changes reflected a decrease in HD lipoprotein cholesterol. The lipid and lipoprotein changes were similar to those taking place during pregnancy; however here the increase of triglycerides was much greater. It was suggested that since the progestins used were 17-alkylated and 19-nortestosterone derivatives and therefore were similar to methyl testosterone derivatives, the effects observed might be due to the progestational or testosterone like component of the anovulatory drug.

The effects of androgens on serum lipids are of relevance in view of the chemical similarity between certain synthetic progestins and methyl testosterone. Androgens usually increase serum LDL and decrease serum HDL levels while serum cholesterol levels remain unchanged (104). However the administration of a combination of synthetic steroids may result in changes which cannot be accounted for by the effects of these steroids administered singly. More recently Wynn et al. (105) reported elevations in several types of lipoproteins resulting from oral contraceptive therapy, i.e., increases in the Sf 0-12, 200-100 and 100-400 but no change in Sf 12-20. Barton et al. (106) surveyed serum cholesterol and triglyceride levels in women taking oral contraceptives at three monthly intervals for two years and found an elevation in both of these values in patients taking both estrogen and progestogen, but no change in those taking progestogen only. The increase was proportional to the size of the dose administered; the elevated lipid levels decreased following the withdrawal of treatment for six months.

Pincus (107) however reported no change in cholesterol levels or in LD lipoproteins in women receiving the oral contraceptive drug Enovid for one year. But Aurell et al. (108) studied the effect of another compound, Anovlar, for one year in eight patients and observed a significant rise in serum lipids, especially in serum LD lipoproteins, similar to the levels typical of post-menopausal women. This elevated level of LD lipoproteins lasted as long as

the drug was administered whereas the hyperlipidemia of pregnancy is seen only during the last two trimesters. The authors suspect either an androgen-like activity of norethisterone or possibly an inhibition of normal ovarian estrogen secretion. Contrary to these results Brody et al. (109) reported no significant changes in plasma triglycerides or cholesterol in two groups of five women receiving Anovlar or Volidan for six months. Glueck et al. (110) showed improvement in type V hyperlipidemia when progestogens were given to women, thus the adverse effects on lipid levels appear to be due to estrogens.

A report by Smith and Prior (111) indicated that Anovlar interfered with the serum cholesterol-lowering action of clofibrate in a 30-year-old woman with familial hypercholesterolemia (type II).

Daily treatment with 5 mg Enovid resulted in similar changes in estrogen-dependent lipid levels (112). Whereas total cholesterol and the LD lipoprotein cholesterol decreased, there was an increase in phospholipids and in HD lipoprotein cholesterol. This effect was not manifest when the daily dose was increased to 10 mg. It was assumed that at the higher dose there was sufficient progestin component to inhibit the changes induced by the estrogenic component.

According to Elgee (113) a combination of peripheral resistance to insulin with hyperinsulinemia may enhance insulin-induced hepatic synthesis and release of VLDL thereby causing hypertriglyceridemia. Progestin alone can decrease hypertriglyceridemia in type V hyperlipoproteinemia.

Sachs et al. (114) reported that the contraceptive drug Ovulen given for periods of 18 months increased plasma triglycerides in women by 76%. They also observed increases in VLDL and in plasma phospholipids and cholesterol. They suggested that a possible increase of apoprotein associated with VLDL might be expected since estrogen therapy is known to be associated with increased serum levels of certain carrier proteins (115). Furman et al. (116) stated that the alpha and beta lipoproteins contain a greater percentage of protein than normal during estrogen therapy and the possibility exists that increased lipoprotein levels during oral contraceptive therapy may be secondary to increased rates of lipoprotein apoprotein synthesis by the liver (117).

When serum lipids were measured in women over a period of 6-60 months of treatment with a combination of mestranol and norethynodrel, it was observed that HDL level increased and remained slightly elevated (118). Serum total cholesterol was not affected whereas triglycerides increased during the first 6-18 months only. Zorilla et al. (119) reported increased serum triglycerides, VLDL and cholesterol during sequential therapy with mestranol and mestranol-chlormadinone acetate combination. When the once a month contraceptive (quinestrol with quingestanol) was administered to nine women for 12-52 weeks it was observed that although plasma cholesterol did not change, both triglycerides and phospholipids increased by 32% and 16% respectively (120). The elevated triglyceride was believed to occur as a result of increased hepatic synthesis. In general changes were qualitatively similar to those occurring as a result of more conventional administration of oral contraceptives.

Decreased FFA levels in plasma of women taking the anovulatory drug Anoncene were observed by Larsson-Cohn et al. (121). This may be due to elevated insulin levels which were reported to occur during contraceptive treatment (122). Insulin reduces FFA concentration (123). However Spellacy et al. (124) and Wynn et al. (103) found elevated levels of FFA in patients treated with oral contraceptives. Changes in insulin activity could possibly stimulate hepatic triglyceride synthesis and secretion into plasma (125,126). Also an alteration in liver function may result in an increased formation or release of triglycerides (127).

Individual plasma phospholipids have been shown to be influenced by female sex hormones, e.g., estradiol, with a reduction especially in the percentage of lysolecithin present (62). Similar changes also have been observed after the administration of anovulatory hormonal compounds (109). In general the concentration of individual phospholipids changes in a manner similar to that found during pregnancy (128).

Even though Eisalo et al. (68) reported that in women changes in liver function tests resulting from oral contraceptives could be attributed to the influence of either estrogen or progestin rather than to their synergistic action, Brody et al. (129) concluded that the overall effects of Anovlar, Ovulen and Volidan reflect competition between their estrogenic and progestogenic components. In these cases norethisterone acetate resulted in an effect on plasma lipids opposite to that induced by estrogen. Although triglycerides were increased by Ovulen and Anovlar administration, phospholipids were decreased by Anovlar and increased by Ovulen and Volidan. When Seng et al. (130) investigated the effects of lynestrol and mestranol on various aspects of fat metabolism, they observed increased fatty acid oxidation in addition to increases in triglycerides, glycerol,

beta-hydroxybutyrate and acetoacetate.

Effect on Platelets. It has been reported that in women receiving oral contraceptives, platelet behavior resembles that of patients with arterial disease in that the sensitivity of platelets to ADP increases (16). Apparently this is the result of the appearance of an abnormal lecithin in LDL in plasma: under the influence of a labile plasma component the lecithin is converted to a substance that affects platelets. The estrogen component of the anovulatory drug is evidently responsible for this effect since progestin alone is inactive.

Although Ham and Rose (131) found no difference in platelet count and adhesiveness, studies of Adams et al. (132) confirm the results previously reported by Bolton and coworkers (16). In subjects from whom paired blood samples were obtained the mean postheparin lipolytic activity was depressed by the administration of combined estrogen-progestin contraceptive preparations (a variety of commercial products). A change in platelet behavior was also observed although this alteration was less striking and less consistent than was the depression of PHLA. Bolton et al. (16) suggested that such behavior was not due to an abnormality in the platelets but rather to an alteration in plasma lipids. However Adams et al. (132) concluded that a correlation between PHLA and platelets behavior did not necessarily exist either before or after oral contraceptive administration.

Effect on Circulating Hormones. It is not known whether the effects of oral contraceptives on serum lipids and lipoproteins are due to a primary effect of estrogen or progestin, or both, or whether the elevated levels of circulating hormones which have been observed then affect the lipid levels. In patients newly exposed to oral contraceptives Spellacy and coworkers (133-135) found an increase in growth hormone levels accompanied by hyperinsulinemia, the latter probably arising as a consequence of the elevated growth hormone. Spellacy et al. (136) believe that observed derangements in carbohydrate metabolism resulting from oral contraceptive administration were mediated through the increased levels of growth hormone wich are known to be diabetogenic. The hyperinsulinemia could be also related to the increased plasma triglycerides or cortisol levels (137).

In view of the hyperinsulinemia reported by several investigators it is not surprising that there are also several reports of clinical improvement of diabetes due to estrogen treatment. Successful treatment of patients with abnormal (diabetic) glucose levels accompanying acromegaly (which results from an excess secretion of growth hormone) with estrogen has been reported by McCullagh et al. (138). Spiegelman (139) noted that insulin requirements decreased by 63% in premenopausal and by 42% in postmenopausal women after estrogen therapy.

Although estrogens seem to protect the diabetic experimental animal and mildly diabetic human, they tend to cause an excess secretion of insulin and growth hormone and an elevated glucose level in normal human beings. It appears that in normal subjects the insulin levels become high enough so that the glucose levels return to their pretreatment range after several months of use. In some subjects the glucose levels rise again with prolonged usage of estrogenic compounds. The effect on glucose metabolism is related to the type and dose of estrogen used (122). Oral contraceptive therapy also was found to increase the activity of adrenal cortex hormones. Thus an increase in biological activity of hydrocortisone was observed by Wynn and Doar (140). Also Williamson (141) reported elevations of plasma cortisol in 72% of oral contraceptive users. Significant increases in plasma cortisol (as well as aldosterone) had been previously observed by Layne et al. in 1961 (142) as a result of Enovid, norethynodrel or mestranol administration.

Cortisol by itself exerts a great influence on several aspects of carbohydrate and fat metabolism. It mobilizes free fatty acids, decreases their reesterification, increases lipogenesis in the liver, increases the mobilization of triglycerides from the liver and promotes greater fatty acid oxidation. At the same time its gluconeogenic effect leads to hyperglycemia which stimulates insulin secretion, which in turn has antilipolytic effect (143). It is obvious therefore that most of the observed metabolic effects of oral contraceptives could be related to an oversupply of cortisol. Finally it has been observed (144) that cholesterol content of the menstrual discharge while in general lower than that of serum is even lower in women using oral contraceptives.

Relationship to Atherosclerosis. Since oral contraceptives have profound effects on lipid metabolism, be it directly or indirectly, and since elevated serum lipid and lipoprotein levels are associated with the development of clinical manifestations of atherosclerosis, it is of great importance to look for changes in susceptibility to this disease in the millions of women to whom these drugs are being administered.

According to Oliver (145) oral contraceptives per se do not seem to increase the risk of developing myocardial infarction in normal

women, although by raising serum lipids, reducing carbohydrate tolerance, raising blood pressure and increasing platelet adhesiveness, they may do so in women prone to ischaemic heart disease. Further studies in this area are needed; continual testing and examinations should accompany administration of oral contraceptives.

Summary. It is obvious that oral contraceptives affect many aspects of lipid metabolism. In all probability the observed metabolic changes are the result of several independent effects.

Since the anovulatory drugs are a mixture of very specific metabolic agents which may at one time act in opposition to and at other times synergistically with each other, the observed effects are sometimes controversial. In addition these exogenous hormone-like substances must act by reinforcing or antagonizing the endogenous individual hormonal balance which increases the incidence of conflicting results. The endogenous hormonal balance may be a characteristic of the individual influenced to a certain degree by age. On the other hand the dose level and the type of the combined drugs may also affect the resulting metabolic changes.

Although not all the investigations produce comparable and confirmatory results, those obtained on lipoprotein distribution and composition appear to be of particular importance. These observations may or may not be a direct consequence of the administration of the estrogen-progestin mixture or may or may not be mediated indirectly through the action of this mixture on other hormones. It is also possible that the action of other hormones in addition to growth hormone, cortisol and insulin is evoked in response to direct metabolic changes caused by the anovulatory mixture.

There is considerable controversy as to whether cholesterol levels are elevated or depressed during the contraceptive therapy; results on cholesterol are dependent on the level and distribution of lipoproteins, since even though the cholesterol content of HDL relative to phospholipid and protein content diminishes, the concentration of HDL increases. Apoprotein synthesis appears to be stimulated as well.

It is generally accepted that triglyceride levels are increased due possibly to changes in lipoprotein lipase or else as a result of increased insulin levels or derangements in liver function. Since liver function is affected by the drugs, it may affect lipoprotein composition and release and thus influence lipid transport.

Obviously studies on animals permit more thorough observations of changes taking place. Although these may be directly applicable to humans, the final analysis and elevation of these drugs must come from human experiments. Little is known about long range effects of oral contraceptives on women and on any progeny born after discontinuation of oral contraceptive therapy. Much more research is needed to define the effects of antiovulatory drugs on lipid metabolism. Long term studies to determine whether the effects of oral contraceptives on lipid metabolism are reversible after their use is discontinued are important to maintain the health and welfare of young women during their reproductive years. Studies on incidence of atherosclerosis in young women need to be initiated to determine whether there is an increased susceptibility or resistance to vascular disease as well as to other hormone-linked diseases, e.g., osteoporosis, diabetes, gout, etc.

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Fatty Acid Synthesis in Rat Testes Injected Intratesticularly or Incubated with 1-^{14}C Acetate [1]

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ABSTRACT

Fatty acid synthesis was studied in testes of young and adult rats either injected intratesticularly or incubated with 1-^{14}C acetate. The pattern of ^{14}C incorporation into lipids and individual fatty acids in the two age groups was similar but results obtained with intratesticular injection differed considerably from those obtained in the in vitro studies. In the former more than 70% of the ^{14}C incorporated in total lipids was in phosphatides, with about 15% in triglycerides and only minor amounts in cholesteryl esters and free fatty acids. Most of the ^{14}C incorporated into total fatty acids was in saturated acids (predominantly 16:0). A small amount of ^{14}C was in the higher polyenes and there was a progressive increase with time after acetate injection in the ^{14}C content of 22:5 W6. In testes incubated with 1-^{14}C acetate, the phosphatide, triglyceride, and free fatty acid fractions had similar amounts of ^{14}C. In the total fatty acid fraction about 40% of the incorporated ^{14}C was in saturated acids (predominantly 14:0 and 16:0) and about 50% in the higher polyenes. Twenty carbon polyenes and 22:5 W6 had significant ^{14}C incorporation, but most of the ^{14}C was in 22:4 W6. About 80% of the ^{14}C in the latter compound was in the carboxyl carbon, indicating its origin from endogenous 20:4 W6 elongated by the added 1-^{14}C acetate used as substrate. The ^{14}C 22:4 was present predominantly in the triglyceride and phosphatide fractions with minor amounts in other lipids. ^{14}C-labeled compounds of retention time greater than 22:5 were also present in all lipid fractions.

[1] Presented at the ISF-AOCS World Congress Chicago, September 1970.

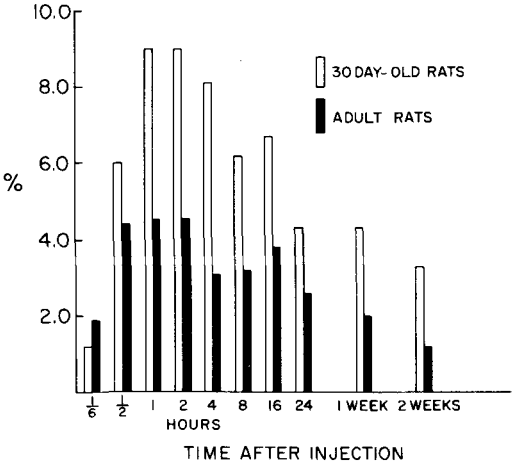

FIG. 1. The amount of ^{14}C found in testicular fatty acids at various time intervals after intratesticular injection of 1-^{14}C-acetate expressed as per cent of the injected dose. Each bar represents the average of results obtained using two different animals and these values usually agreed within 15%.

INTRODUCTION

Rat testicular tissue is quite active in the synthesis and interconversion of fatty acids (1-3). Because of this active metabolism and the accessibility of this organ for direct introduction of substrate, it is a useful system for the study of lipid metabolism. In this paper we report some experiments in which we compare the incorporation of ^{14}C into fatty acids and lipids in rat testes injected intratesticularly with those incubated with ^{14}C-acetate. The results obtained by these two methods differed considerably and both may be useful for studying the lipid metabolic activities of this organ.

EXPERIMENTAL PROCEDURES

Sprague-Dawley rats of 30 days of age (young rats) or of from six months to one year of age (adult rats) maintained on Purina laboratory chow were used in the in vivo studies. The rats were injected intratesticularly with .05 ml of a water solution of 1-^{14}C sodium acetate containing 5 μc and less than 0.1 mg acetate and killed at various time intervals as indicated. For the in vitro studies, testes were removed and chilled in ice immediately after the rats were decapitated. After removal of the *tunica albuginea* each testis was weighed and divided into four approximately equal pieces which were placed in flasks containing the appropriate

TABLE I

Weight of Testes and Amounts of Testicular Fatty Acids in Young and Adult Rats Injected Intratesticularly with ^{14}C Acetate

Age of rats	Number of rats	Weight of testes, g[e]	Total fatty acids, mg/g testes
4 weeks[a]	16	0.64 (0.56-0.76)	10.1 (8.4-12.0)
5 weeks[b]	2	1.23 (1.02-1.44)	9.1 (8.5-9.6)
6 weeks[c]	2	1.56 (1.48-1.64)	11.3 (10.2-12.4)
6-12 months[d]	20	3.52 (3.20-3.91)	11.4 (9.5-14.4)

[a]Includes all young rats of time periods from one sixth through 24 hr after injection.
[b]Includes young rats of time period one week after injection.
[c]Includes young rats of time period two weeks after injection.
[d]Includes adult rats of all time periods.
[e]Average and range of values.

incubation medium and substrate. One testis of an adult animal was used per flask. In the case of young animals multiple testes were used in one flask in order to approximate the weight of one adult testis. The following medium was used: 0.20 ml of a water solution containing 10 μc (~0.2 mg) 1-^{14}C sodium acetate, 0.50 ml of 1.0 M phosphate buffer (pH 7.4), 1.30 ml of 10 mM glucose, and distilled water to make a total of 2.6 ml. Incubations were at 37 C for 3 hr and the gas phase was air. In these experiments the young rats were either four or seven weeks of age; adult rats were between six and twelve months of age. Incubations were terminated by addition of potassium hydroxide to those samples which were to be hydrolyzed for subsequent isolation of total fatty acids or by pouring the reaction contents into Folch mixture in the case of the samples in which total lipid was extracted. Procedures for extraction and separation of lipids, hydrolysis of tissues and lipids, extraction of fatty acids, gas chromatographic and gas radiochromatographic procedures have been described previously (2,4). Procedures for isolation, complete identification, and degradation of docosatetraenoic acid were, likewise, similar to those used previously for other higher polyenes (2). Radioactivity determinations of isolated lipids and fatty acids were made in a liquid scintillation spectrometer operating at 80% efficiency.

RESULTS

In Vivo Experiments

In adult rats the amount of ^{14}C found in testicular fatty acids increased from about 1.9% of the injected dose at 10 min after injection to about 4.5% at 30-120 min (Figure 1). Thereafter it decreased to 2% and to 1.5% at one and two weeks, respectively. In the young rats the amount of ^{14}C in testicular fatty acids increased from about 1% of the injected dose at 10 min to about 9% at 1-2 hr; thereafter it decreased to about 3% at the end of two weeks. The results shown in Figure 1 are averages of values obtained for two different rats during each time interval. In about 75% of the cases the two values agreed within 15%, but variations up to 30% were encountered. In spite of these variations there apparently was a significant difference between young and adult animals in the amount of ^{14}C retained in total fatty acids at most of the time periods studied. This is more apparent if the weights of the

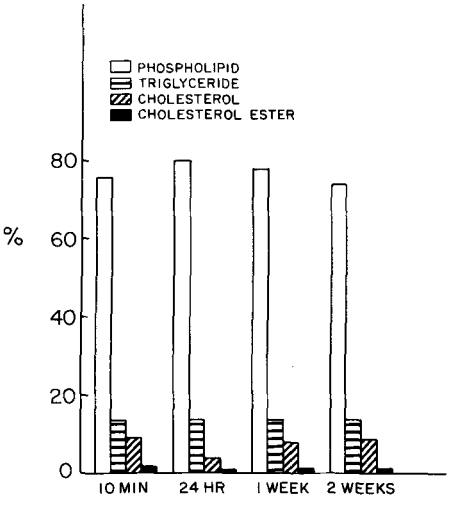

FIG. 2. The amount of ^{14}C found in various lipid classes of adult rat testes at several time intervals after intratesticular injection of 1-^{14}C-acetate expressed as per cent of the total counts eluted from the chromatogram. Recovery of counts spotted on the chromatograms was greater than 85%. Values for young animals were similar to those of the adults. Significant quantities (1-3%) of ^{14}C in the free fatty acid fraction were found only in time intervals up to 1 hr.

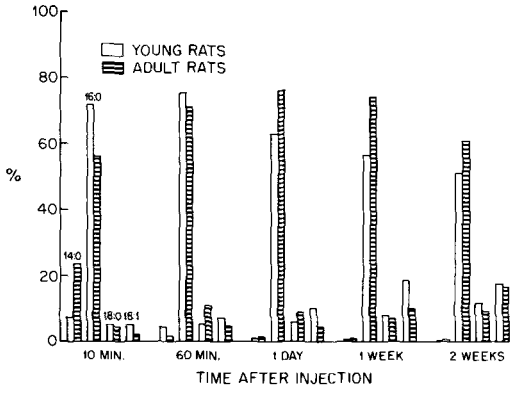

FIG. 3. The distribution of ^{14}C in the total saturated and monoenoic acids of adult and young rats at various time intervals after intratesticular injection of 1-^{14}C-acetate. Results are expressed as per cent of the total ^{14}C eluted from the column and for each component are averages of two different animals per time period.

testes of young and adult rats, shown in Table I, are considered and the data expressed as per cent of administered dose retained per gram of tissue. Although the weights of testes of young and adult rats were markedly different, there was little or no difference in the concentrations of total fatty acids (mg/g testis, Table I).

The distribution of the ^{14}C in various lipid fractions was determined after separation of the lipid classes by thin layer chromatography (TLC) using silicic acid, and the results are shown in Figure 2 for adult rats at several time periods. Values obtained for the young animals were similar to those of the adult animals. Most of the activity (>70%) was in the total phosphatide fraction at all time periods with the triglyceride fraction having the next highest activity (about 15%). Small amounts were found in the cholesteryl ester fraction and significant quantities (1-2%) in the free fatty acid fraction only in the time periods up to 1 hr. Only traces of activity were found in the free fatty acids fraction in time intervals after 1 hr. Recovery of ^{14}C from thin layer plates in experiments reported in this paper was always greater than 85% of the ^{14}C activity applied to the plates.

The distribution of the ^{14}C in individual fatty acids of the total fatty acid fraction was determined using a continuous flow radioactivity monitor (heated proporational counter) attached to a gas liquid chromatograph. Identification has been made for most components by relative retention time using standards. Some components for which standards are not available were identified in previous work by use of chemical procedures (2,5). In Figure 3 are given results of the ^{14}C in saturated acids and in monoenoic acids, expressed as per cent of the total fatty acid ^{14}C in the total chromatogram. The values plotted are the averages of the two animals per time interval. In both young and adult animals most of the ^{14}C was found in palmitic acid with some decrease occurring by the end of the second week after injection. Activity in 14:0 was significant only in the short time intervals (1 hr or less after injection). The amount of ^{14}C in 18:0 and 18:1 increased with time. Results of ^{14}C in higher polyenes have been grouped in five sections because of overlap of some peaks and for convenience of presentation (Fig. 4). A significant incorporation into 18:2 in practically all time periods

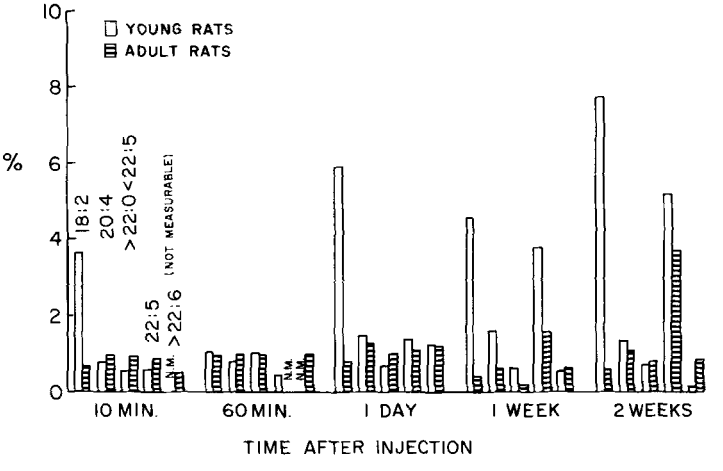

FIG. 4. The distribution of ^{14}C in the higher polyenes of the total fatty acid fraction of adult and young rats at various time intervals after intratesticular injection of 1-^{14}C-acetate. Results are expressed as for Figure 3 and they are averages of two animals per time period.

TABLE II

Distribution of ^{14}C in Lipid Classes of Adult Rat Testes Incubated with 1-^{14}C-Acetate

Lipid fraction	Per cent of total ^{14}C eluted from chromatogram[a]
Phospholipid	30
Diglyceride	3
Cholesterol	7
Free fatty acid	25
Triglyceride	30
Cholesteryl ester	5

[a]Recovery of ^{14}C activity from eluted spots was greater than 85% of the ^{14}C activity applied to plate. Data are averages of two different determinations which agreed within 10%.

occurred in young rats but not in adults. This fraction was not further characterized but presumably it is the $\Delta^{6,9}$ 18:2, since the $\Delta^{9,12}$isomer (linoleic acid) should not be labeled. These two isomers elute at the same time under our conditions of gas radiochromatography. The total amount of incorporation into all higher polyenes is not large for either young or adult rats, but there was a progressive increase with time after injection in the ^{14}C incorporated into the 22:5 fraction. Not enough ^{14}C activity was present in the 24 carbon polyenes to detect by this method of radiochromatography.

In Vitro Experiments

Total incorporation of ^{14}C from 1-^{14}C-acetate in 3 hr of incubation was about 5% of the substrate per gram of testis for both young and adult animals. The concentration of fatty acids in testes of these animals was not determined, but in experiments done similarly to these using testes from two 4-week-old rats and two 6-months-old rats (one determination per each testis) the concentrations of total fatty acid were similar to those reported in Table 1 for the in vivo experiments (9.1-10-7 mg/g for 4-week-old and 12.4-13.0 mg/g for 6-month-old rats). In Table II is shown the distribution of ^{14}C in various lipid classes for adult rats. It is evident that the pattern is far different than that seen after intratesticular injection of ^{14}C acetate. In the in vitro studies only about 25% of the activity was found in the phosphatide fraction with similar amounts in triglyceride and in free fatty acids. Other fractions had small but significant amounts of ^{14}C activity. A similar distribution of ^{14}C in lipids of testes of young animals was observed.

The distribution of ^{14}C in total fatty acids of the incubated testes of rats of various ages is shown in Figure 5. About half of the activity in

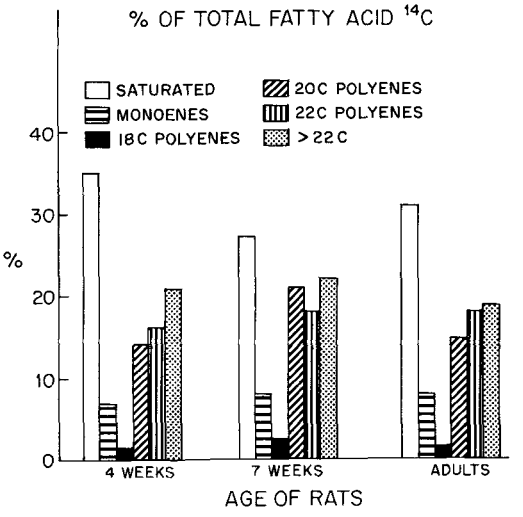

FIG. 5. The distribution of ^{14}C in the total fatty acids of testes of adult and young rats incubated with 1-^{14}C-acetate. Incubation conditions and further identifications of the various fractions are given in the text. Results are expressed as for Figure 3.

the saturated fraction was in 16:0, one third in 14:0, and one sixth in 18:0. The activity in the monoene fraction was essentially all in 18:1. Activity in the 20C polyene fraction was spread rather uniformly in several peaks, including 20:2, 20:3, 20:4, and perhaps 20:5. At least two thirds of the activity of the 22 carbon polyene fraction was in a compound identified by relative retention time as 22:4, while the remainder was in the 22:5 fraction. Because of the unusually large incorporation into the compound identified as 22:4, the pure compound was isolated from pooled samples and purified to greater than 99% radiopurity as evaluated by gas radiochromatography. Hydrogenation of the compound and analysis of the hydrogenated derivative indicated that all the ^{14}C was in a 22 carbon acid. The original compound was isomerized at 180 C in 21% potassium hydroxide for 20 min and the resulting material had a maximum ultraviolet absorption band at 315 mμ indicating that there were four double bonds. Finally oxidative ozonolysis yielded a radioactive 7 carbon dicarboxylic acid, indicating that the first double bond from the carboxyl group was seven carbons away. Thus, the structure of the material was shown to be $\Delta^{7,10,13,16}$ 22:4. ^{14}C compounds with a retention time greater than 22:5 were grouped together, and as shown in Figure 5, a considerable amount of ^{14}C was incorporated into this group. Although detailed identification of these has not yet been done, they have been hydro-

TABLE III

Distribution of ^{14}C-Fatty Acids in Lipid Fractions of Adult Rat Testes Incubated with 1-^{14}C-Acetate

	Per cent of total ^{14}C eluted from chromatogram			
Fatty acid	Triglycerides	Phosphatides	Cholesteryl esters	Free fatty acids
14:0	20.0	12.5	10.0	16.2
16:0, 16:1	15.0	30.5	8.8	26.5
18:0, 18:1	2.5	19.2	2.0	14.3
20:2, 20:3, 20:4	10.1	4.0	8.9	1.3
22:4	21.8	12.7	44.0	18.2
22:5	5.2	4.7	6.1	0.8
> 22:5	20.7	13.3	14.2	20.2

genated and shown to be predominantly 24 carbon compounds. On the basis of relative retention time it is suggested that 24:4 and 24:5 are two of the components.

Chemical degradation of the isolated ^{14}C 22:4 after hydrogenation to 22:0 showed that most of the ^{14}C activity was in the carboxyl carbon, the ratio of specific activities of carboxyl carbon to average carbon being 16.

It was of interest to determine in which lipid fractions the ^{14}C 22:4 was incorporated. Therefore gas liquid radiochromatographic analyses were done of fatty acids obtained after hydrolysis of various lipid classes separated by TLC. The results, expressed as per cent of the total counts eluted from the chromatogram, are summarized in Table III. The largest concentration of ^{14}C 22:4 was in cholesteryl esters. However quantitatively this is not significant since the cholesteryl ester fraction is a minor component of the total lipid in testis. A larger concentration of ^{14}C 22:4 was in triglycerides and in free fatty acids than in phosphatides. Free fatty acids also represent a minor component of total lipid so that a significant percentage of the ^{14}C 22:4 in the testis occurs in triglycerides. In contrast to the 22:4, ^{14}C 22:5 was uniformly distributed in these fractions, except for free fatty acids. Of considerable interest also is the large proportion of the ^{14}C in compounds of retention time greater than 22:5 that is in triglycerides and the detection of these labeled compounds in cholesteryl esters and in free fatty acids.

DISCUSSION

Results obtained in the experiments using intratesticular injection of ^{14}C acetate were quite different from those obtained in incubation experiments. Not only was the distribution of ^{14}C in lipid classes different in the two types of experiments, but also the pattern of ^{14}C labeling in the fatty acids was different. The mechanisms which are responsible for the observed differences are probably multiple and complex. These include hormonal and nervous influences acting in the intact animal as well as accessibility of substrate in each case, possible faster deterioration of the incubated tissue, differences in transport in the two cases not only of the ^{14}C substrate but also of the synthesized fatty acids and differences in rates of related reactions such as esterification.

The possibility that ^{14}C fatty acids were made in organs other than the testes and transported back to this organ in the in vivo experiments is considered negligible for the following reasons: In other experiments (not published) 20 μc of 1-^{14}C-acetate were injected intraperitoneally into rats and after 24 hr less than .03% of the administered dose was incorporated into testicular fatty acids; 4 hr after intratesticular injection of 1-^{14}C-linoleate into one testicle of a rat the other testicle of the same rat had only 0.3% of the total ^{14}C recovered in the injected testicle. About 7% of the ^{14}C activity of testicular total fatty acids in these experiments was in palmitic acid, indicating that a significant part of the labeled linoleate had been catabolized to 2 carbon fragments which were then available for de novo synthesis in the testis or transport to other organs (4).

If it is assumed that the metabolism of the ^{14}C-acetate after intratesticular injection follows the "more physiological" pathway, in vitro experiments may still not only be useful but also necessary to allow modification of conditions and opportunity for events to be observed which otherwise might be masked in the intact animal. Therefore it appears that both types of experiments yield useful information. In the intact animal ^{14}C-acetate was used mostly for formation of saturated acids, but as time progressed the longer chain, more highly unsaturated fatty acids had relatively increased amounts of ^{14}C. In the incubation experi-

ments, under these conditions, elongation of endogenous fatty acids by the added ^{14}C-acetate formed a significant portion of the over-all incorporation with the predominant products being polyenes of 20,22 and 24 carbons. The large ^{14}C activity in the 22:4 fraction indicates that arachidonic acid in the testis is available to the enzymes responsible for elongation with added ^{14}C-acetate. That the formation of the ^{14}C 22:4 was apparently a simple elongation of endogenous arachidonic acid was proven by chemical degradation of the isolated pure ^{14}C 22:4. About 80% of the activity in the whole molecule was in the carboxyl carbon. Although the polyenoic acids are thought to be important in membrane structures through their association with phospholipids, much of the labeled 22:4, 22:5 and labeled components of retention time greater than 22:5 was found in triglycerides. The latter probably included the 24:4 and 24:5 described recently by Bridges and Coniglio (2). Although in a recent report (6) the statement is made that these occur exclusively in the triglyceride fraction of total lipid isolated from testis, much of the labeled 24:4 and 24:5 in the in vitro experiments reported here was found in the phospholipid fraction. In our experiments the total phosphatide fraction was not fractionated into individual types of phosphatides. Morin (7) had previously reported that phosphatidyl choline incorporated more ^{14}C than phosphatidyl ethanolamine or sphingomyelin when slices of rabbit testes were incubated with ^{14}C-acetate. The significance of the large amount of ^{14}C 22 and 24 carbon polyenes in triglycerides is not known, but these may represent transport forms of these polyenes. Incubation studies will be useful in elucidating the sequence and controls of the reactions which are responsible for these products.

ACKNOWLEDGMENTS

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Occurrence of *Cis*-6-Hexadecenoic Acid as the Major Component of *Thunbergia alata* Seed Oil[1]

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ABSTRACT

An unusual series of monoenoic fatty acids constitutes about 85% of the total acids in seed oil from *Thunbergia alata*. The major component in the oil, *cis*-6-hexadecenoic acid (82%), is accompanied by the homologous 4-tetradecenoic (ca. 0.2%) and 8-octadecenoic (1.8%) acids. Another homologous series is represented by 5-tetradecenoic (ca. 0.2%), 7-hexadecenoic (1.8%) and the familiar 9-octadecenoic (4.4%) acids. Traces (<0.1%) of three other acids, 6-tetradecenoic and 10- and 11-octadecenoic, are also present along with palmitic (5.8%), stearic (0.6%) and linoleic (2.2%) acids. Some of the monoenoic acids have not previously been known to occur in seed oils.

INTRODUCTION

Members of the genus *Thunbergia* (family Acanthaceae) are mostly tall perennial climbers widely grown in greenhouses or in the open air in warm climates. *Thunbergia alata* Boj. ex Sims (Black-eyed Susan, but not to be confused with the nonviny *Rudbeckia* spp. also called black-eyed Susan) is often grown as an annual garden plant which flowers in the late summer (1). Gas liquid chromatography (GLC) of *T. alata* seed oil and the methyl esters prepared from it indicated that almost 90% of the acids in the oil contained 16 carbon atoms. Since these acids rarely constitute such a large proportion of a seed oil, the further characterization studies reported here were undertaken.

[1]Presented at the Fifth Great Lakes Regional Meeting of the American Chemical Society, Peoria, Illinois, 1971.

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TABLE I

Analytical Data on *Thunbergia alata* Seed and Oil[a,b]

Component	% by GLC
14:0	0.1
14:1[4], 14:1[5], 14:1[6]	0.4
15:1	0.1
16:0	5.8
16:1[6]	82.2
16:1[7]	1.8
17:1	0.2
18:0	0.6
18:1[8]	1.8
18:1[9]	4.4
18:2[9,12]	2.2
18:3	0.1
20:0	0.1
20:1	Trace
22:0	0.1

[a]Seed weight, 25 g/1000 g; per cent of oil, 20 db; per cent of protein, 24 db (N x 6.25); HBr equivalent as epoxyoleic acid, 0.7%; refractive index, 1.4625 n_D^{40}; iodine value, 88.7 Wijs, 88.8 calculated.

[b]Fatty acid composition based on area percentages of methyl esters; percentages of positional isomers based on peak areas of ozonolysis fragments (5).

EXPERIMENTAL PROCEDURES

Oil was extracted from ground seed with petroleum ether (bp 30-60 C) and was analyzed by direct GLC in a manner similar to that of Litchfield et al. (2). Methyl esters were prepared from the oil (3) and their equivalent chain lengths were determined by GLC (4) on a Packard 7401 gas chromatograph. Esters were fractionated by chain length by preparative GLC on an Autoprep A-700 gas chromatograph equipped with a 6 ft x 1/4 in. stainless steel column packed with 20% Apiezon L on 60/80 mesh Celite 545. In the column held at 225 C, helium served as the carrier gas at a rate of 180 ml/min. To minimize losses, the collection device on the chromatograph was modified by replacing the collector tip with a 1/4 in. brass, male, Swagelock fitting silver-soldered to the outlet. Fractions were collected in 8 in. x 1/4 in. O.D. glass tubes packed with Adsorbosil CAB and attached to the fitting with 1/4 in. Teflon ferrules and brass nuts.

Preparative thin layer chromatography (TLC) was performed on plates spread with a 0.25 mm layer of Silica Gel G containing 20% $AgNO_3$. The developing solvent was a 50:50 mixture of C_6H_6 and $CHCl_3$. After development the plates were sprayed with an alcoholic solution of 2',7'-dichlorofluorescein so that the bands could be observed under ultraviolet (UV) light. The bands were scraped from the plates and the esters were recovered from the adsorbent with ether.

Combinations of ozonolysis and GLC (5) and of GLC and mass spectrometry (MS) were used to locate double bonds. In the GLC-MS combination, methoxy derivatives were pre-

pared (6) from the mixed esters. These derivatives were introduced into a CEC 21-492-1 mass spectrometer by a GC inlet system (jet sample enricher) from a Packard 7401 gas chromatograph equipped with a 6 ft glass column packed with 3% OV-101 on Gas Chrom Q. The column oven was held at 200 C and the source of the mass spectrometer at 210 C. Infrared (IR) spectra of the samples were recorded on a Perkin-Elmer Model 137 spectrophotometer either from liquid films (sodium chloride disks) or from carbon disulfide solutions (1 mm sodium chloride cells).

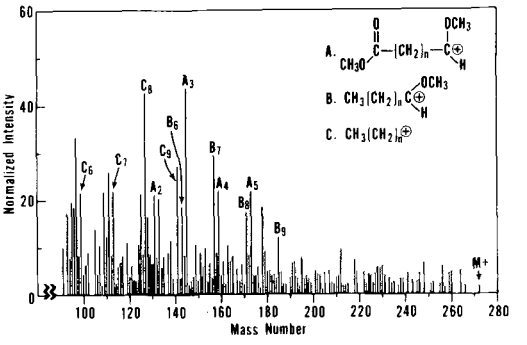

FIG. 1. Mass spectrum of methyl methoxytetradecanoates from *Thunbergia alata* esters by gas liquid chromatography-mass spectrometry. Fragment type is denoted by letter with subscript indicating the number of carbon atoms. Peaks below mass 90 are not essential to the identification of the esters and are not shown.

RESULTS AND DISCUSSION

GLC of *T. alata* oil indicated a range of triglycerides from C_{46} to C_{56} with the major peak at C_{48}. Over 98 (area) per cent of the material eluted from the column was included in the triglyceride region of the curve. IR and UV spectra of the oil were consistent with those of normal seed oils. GLC analysis of the methyl esters prepared from the oil indicated that almost 90% of the fatty acids were 16 carbon acids; small amounts of acids containing 14, 15, 17, 18, 20 and 22 carbon atoms were also in evidence.

Identification of Fatty Acid Esters

Preparative GLC yielded three fractions: C_{14}, C_{16} and C_{18} esters. These fractions were analyzed by GLC to determine the esters present. C_{16} and C_{18} fractions were essentially free of contamination by esters of other chain lengths. The C_{14} fraction was small and contained 20% of other esters carried over during collection and about 20% of BHT that had been concentrated from the ether used to recover the esters (7). IR analysis of the C_{16} and C_{18} fractions showed them to be free of *trans* unsaturation.

The C_{18} fraction was further separated according to degrees of unsaturation by preparative TLC on silver nitrate-impregnated plates. The saturated, monoenoic and dienoic esters were collected, and their purity was again established by GLC. The trace of trienoic ester (Table I) was not recovered.

Since the C_{14} and C_{16} fractions contained essentially no polyunsaturated esters by GLC, these fractions were subjected to ozonolysis-GLC without further fractionation. Of the aldehyde-ester (AE) and aldehyde (A) fragments produced from the C_{16} fraction, C_6 AE and C_{10} A made up 98%. Trace amounts of C_7 AE and C_9 A were also found. This evidence established that the major hexadecenoate had *cis*-6-unsaturation and that there was a small amount of 7-hexadecenoate. The C_{18} monoene fraction gave essentially four peaks totaling 96 area per cent from ozonolysis-GLC. These were C_9 AE, C_9 A, C_8 AE and C_{10} A. The fragments define the parent structures as $18{:}1^9$ and $18{:}1^8$. Traces of fragments which could have arisen from $18{:}1^{10}$ and $18{:}1^{11}$ were also found. The only component found in the C_{18} diene fraction was methyl linoleate.

Ozonolysis of the C_{14} fraction did not provide a clear-cut definition of the parent esters because the large amounts of impurities contributed fragments which were indistinguishable from those from the C_{14} esters. Combinations of the fragments indicated that a $14{:}1^4$, a $14{:}1^5$ and a $14{:}1^6$ could be present. Some of the AE and A formed from these esters also could have come from the 18:1 and 16:1 known to be present in this fraction. Nevertheless, the identities of the C_{14} esters were established by MS of their methoxy derivatives (6). Combined GLC-MS, applied to *T. alata* esters, showed strong peaks for the 4-, 5-, 6- and 7-methoxytetradecanoates (Fig. 1). Although these derivatives could come from only a $14{:}1^4$ and $14{:}1^6$, the relative intensities of the peaks together with ozonolysis results strongly suggest the presence also of a $14{:}1^5$. Reliable quantitative data for the C_{14} monoenes were not obtained. Indications were, however, that the $14{:}1^4$ was the most abundant followed by $14{:}1^5$ and then $14{:}1^6$. Spectra from the methoxyhexadecanoates and methoxyoctadecanoates were also consistent with ozonolysis-GLC results.

Total fatty acid composition and some characteristics of *T. alata* oil are given in Table I. Excellent agreement between the iodine value

derived by chemical means (Wijs) and that calculated from the fatty acid composition indicates that the amounts of unsaturated acids given are substantially correct.

According to the latest review of unusual fatty acids in plants (8), *cis*-6-hexadecenoic acid was not known to exist in seed oils. Since this compilation it has been found as a minor constituent of *Picramnia sellowii* (9) and *Beauprea balansae* (10) seed oils and now as a major constituent in *T. alata* oil. Although the unusual tetradecenoic acids have not heretofore been found in seed oils (8), *cis*-8-octadecenoic acid was recently discovered in many species of the Proteaceae (10).

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Fluorescent Products of Lipid Peroxidation of Mitochondria and Microsomes

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ABSTRACT

Liver microsomes and mitochondria and heart sarcosomes from rats fed diets with varying α-tocopherol concentrations and lipid contents were peroxidized over a 6 hr time period. Lipid peroxidation was measured by absorption of oxygen, production of thiobarbituric acid (TBA) reactants and by development of fluorescence. The spectral characteristics of the fluorescent compounds were the same for all peroxidizing systems; the excitation maximum was 360 nm and the emission maximum was 430 nm. As time of peroxidation increased, uptake of oxygen and production of fluorescent compounds increased. These two parameters as well as production of TBA reactants were dependent upon dietary antioxidant and all three had an inverse relationship with the amount of dietary α-tocopherol. The relationship between absorption of oxygen and development of fluorescent compounds was also dependent upon dietary polyunsaturated fats (PUFA). Subcellular particles from animals fed higher levels of PUFA produced more fluorescent products per mole of oxygen absorbed than did those from animals on a diet with lower PUFA content. TBA reacting products increased with time during the initial phase of peroxidation; in the microsomal systems their production stabilized or decreased by 4-6 hr of peroxidation. Using the synthetic 1-amino-3-iminopropene derivative of glycine as standard for quantitation of fluorescence, the molar ratios of oxygen absorbed per fluorescent compound produced were calculated. This ratio for subcellular particles isolated from rats fed diets with PUFA ratios similar to those in the average American human diet was 393:1. The fluorescent compounds had the same spectral characteristics as the lipofuscin pigment that accumulates in animal tissues as a function of age, oxidative stress or antioxidant deficiency. The fluorescent molecular damage represented by that accumulated in human heart age pigment by 50 years of age was calculated to have been caused by approximately 0.6 μmole of free radicals per gram of heart tissue.

INTRODUCTION

The relatively large amounts of polyunsaturated fatty acids (PUFA) in the phospholipids of mitochondria and microsomes (1) cause these organelles to be highly susceptible to lipid peroxidation. In previous studies (2,3) the course of peroxidation of mitochondria was followed by measurements of oxygen absorption and thiobarbituric acid (TBA) reactants and decrease in succinoxidase activity. These studies showed that lipid peroxidation can be inhibited by the lipid antioxidants α-tocopherol and ubiquinol.

Malonaldehyde has been shown to be an important product of oxidized methyl arachidonate and methyl linolenate (4,5). Enzymes with lysine residues such as ribonuclease A are inactivated and crosslinked by malonaldehyde with the production of fluorescent products (6). Fluorescent products derived from malonaldehyde and amino acids have been synthesized and characterized (7). The fluorescence characteristics of products formed during lipid peroxidation of subcellular organelles in vitro were studied previously (8) and the production of these products was shown to be a function of time of peroxidation. The fluorescent products measured are a family of compounds having the structure -N=C-C=C-N- (7,9,10). Fluorescent compounds with the same spectral characteristics have been extracted from age pigments (8,10). Previously only histochemical studies using fluorescence microscopy or staining techniques have been available to study these pigments. Lipid peroxidation in vivo has been identified as a basic deteriorative reaction involved in a variety of oxidative conditions (11-17).

This paper describes studies of lipid peroxidation in liver microsomes and mitochondria and heart sarcosomes from rats fed various levels of α-tocopherol. This in vitro lipid peroxidation system provided a biochemical model for the production of fluorescent compounds and for the determination of the quantitative relationships among absorption of oxygen, production of TBA-reactants and development of

fluorescence. These relationships were found to be related to dietary PUFA and their protection by α-tocopherol obtained through dietary sources.

EXPERIMENTAL PROCEDURES

Animals and Diets

Male Sprague-Dawley rats were placed on their respective diets at four weeks of age. The basal diet (Nutritional Biochemicals Corp.) was similar to the tocopherol deficient diet described by Draper et al. (18). The diet was composed of 10% tocopherol-stripped lard, 65% glucose, 20% casein, 4% salt mixture, and all necessary vitamins except for tocopherol; cod liver oil was added to a concentration of 1% of the diet. This basal tocopherol deficient diet was fed to one group of rats and the same diet supplemented with either 10.5 or 45 mg of dl-α-tocopherol acetate (Nutritional Biochemicals Corp.) per kilogram of diet was fed to two other groups of rats. A fourth group of rats was fed a basal rat chow diet (Purina) for approximately 16-20 weeks. This diet contained 5% fat and 23% protein; the α-tocopherol content was 66 mg/kg of diet.

Preparation of Liver Mitochondria and Microsomes and Heart Sarcosomes

Animals maintained on the above diets for five to six weeks were fasted for 20 hr, decapitated and exsanguinated. The mitochondria and microsomes were prepared from the pooled livers as described by Ragab et al. (19). Heart muscle sarcosomes were prepared from rats fed the tocopherol deficient diet for 14 weeks. The procedure followed was that of Cleland and Slater (20) with saline-versene (0.12 M KCl, 0.02 M phosphate and 0.01 M EDTA, at pH 7.4) as the suspending medium. The final pellets were resuspended in 0.15 M KCl and stored at -15 C. Protein in the above subcellular fractions was determined by the method of Miller (21) with bovine albumin as a standard.

Analytical Methods

Peroxidation of microsomal, mitochondrial and sarcosomal membranes was measured by the Warburg manometric method in an oxygen atmosphere at 37 C. A separate reaction flask was used for each time interval at which parameters of peroxidation were measured. The 4 ml reaction mixtures consisted of 15 mg of subcellular particle protein in a final concentration of 75 mM potassium phosphate buffer, pH 7.04, 1 mM ascorbic acid, 1 mM $FeCl_3$ and 10 ppm chlorotetracycline. Control reaction mixtures were incubated under nitrogen in the absence of initiators of peroxidation. Measurements of oxygen uptake as well as the following assays were made in duplicate.

Fluorescence was measured as follows: aliquots of 0.5 ml of the reaction mixtures were extracted at room temperature with 3 ml of chloroform-methanol (2:1 v/v) by mixing for 1 min on a vortex mixer at high speed. The extracts were centrifuged for 1-2 min after mixing briefly with 3 ml of water. To 1.0 ml of the chloroform layer was added 0.1 ml of methanol and the fluorescence and excitation spectra were determined with an Aminco-Bowman spectrophotofluorometer (American Instrument Co., Inc.) calibrated with quinine sulfate. The slit arrangement for recording fluorescence spectra was slits 3, 4 and 6 set at 3, 1 and 3 mm, respectively. The sensitivity setting was at 50. Spectra were recorded on an X-Y recorder (Houston Instrument). Under these instrument parameters 1 μg of quinine sulfate/ml of 0.1 N H_2SO_4 had a fluorescence intensity of 60 at a 0.3 meter multiplier setting.

The TBA test was done according to the method of Wills (22). Aliquots of 0.2 ml of the reaction systems were added to 1.8 ml of water and 1 ml of 20% (w/v) trichloroacetic acid. Immediately 2 ml of 0.67% (w/v) 2-thiobarbituric acid (Sigma) was added and the tubes were placed in boiling water for 10 min. The absorbance of the cooled and centrifuged samples was read at 532 nm; malonaldehyde was used as a standard.

Mitochondrial succinoxidase was measured polarographically. To the cell thermostated at 25 C was added 2 ml of buffer, pH 7.4 (0.34 M sucrose, 15 mM potassium phosphate, 7.5 mM $MgCl_2$ and 30 mM KCl), 1.45 ml water and 0.5 ml of the lipid peroxidation reaction mixture. After 3 min of equilibration 0.05 ml of 0.2 M disodium succinate was added and oxygen uptake was recorded for 5-10 min. Microsomal DPNH cytochrome c reductase was measured spectrophotometrically by the reduction of cytochrome c at 550 nm (23).

RESULTS

Development of Fluorescence During Peroxidation

In Figure 1 are shown typical fluorescence spectra of the products found in the chloroform phase of the chloroform-methanol extracts of liver mitochondria and microsomes peroxidized for 0, 1, 2 and 4 hr. These spectra with excitation and emission maxima at 360 nm and 430 nm, respectively, are typical of the fluorescence that developed in the reaction systems of all subcellular particles used in these

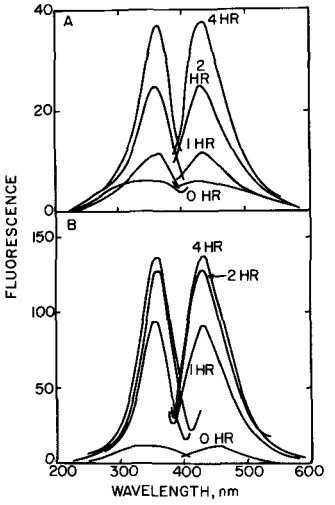

FIG. 1. Excitation and fluorescence spectra of (A) 1.9 mg mitochondria and (B) 1.6 mg microsomes from rats fed 10.5 mg α-tocopherol per kilogram of diet and peroxidized over a 4 hr time period. Excitation, 360 nm; emission, 430 nm; and meter multiplier, 0.03.

studies. The fluorescence in reaction mixtures incubated under nitrogen (not shown in the figure) was qualitatively and quantitatively similar to the zero time samples.

Effect of Dietary α-Tocopherol on the Time Course of Oxygen Absorption and Development of Fluorescence

Figure 2 shows that as the time of peroxidation of mitochondria and microsomes increased (A) the uptake of oxygen and (B) the production of fluorescent compounds increased. The mitochondria and microsomes from animals on the diets that contained stripped lard with a supplement of 1% cod liver oil peroxidized at rates dependent upon protection afforded the PUFA by dietary antioxidant. No peroxidation occurred within 6 hr in mitochondria from animals fed 45 mg of α-tocopherol per kilogram of diet; mitochondria from animals on the tocopherol deficient diet peroxidized at the greatest rate. Microsomes are more labile than mitochondria and those from animals fed the highest level of α-tocopherol did absorb oxygen but at a rate lower than microsomes from animals in the other two dietary groups. The development of fluorescence in the peroxidizing subcellular particles followed the same pattern, the amount of fluorescence having an inverse relationship with the amount of dietary α-tocopherol.

Relationship Between Fluorescence Development and Oxygen Absorption

For all subcellular particles used in these

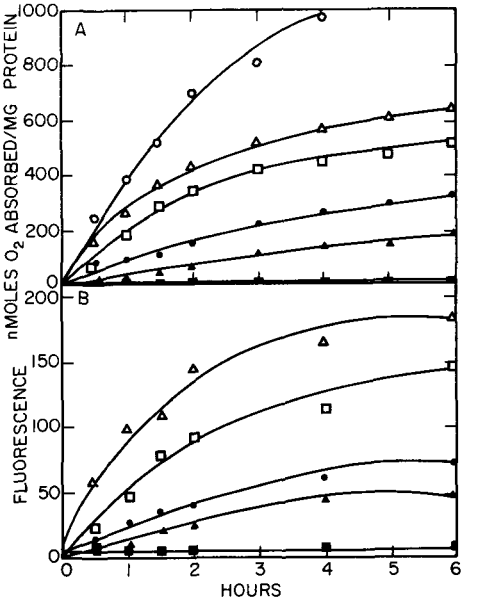

FIG. 2. Effect of dietary α-tocopherol on (A) absorption of oxygen and (B) development of fluorescence. Microsomes from rats fed (○) tocopherol deficient diet, (△) 10.5 mg α-tocopherol per kilogram of diet, (□) 45 mg α-tocopherol per kilogram of diet; mitochondria from rats fed (●) tocopherol deficient diet, (▲) 10.5 mg α-tocopherol per kilogram of diet, and (■) 45 mg α-tocopherol per kilogram of diet. Instrument parameters for fluorescence measurements were meter multiplier, 0.03; excitation, 360 nm; emission 430 nm; slits and sensitivity settings are indicated in the text.

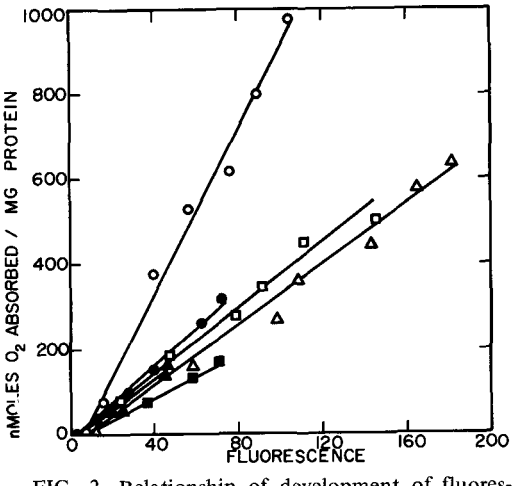

FIG. 3. Relationship of development of fluorescence to absorption of oxygen. Microsomes from rats fed (△) 10.5 mg α-tocopherol per kilogram of diet; (□) 45 mg α-tocopherol per kilogram of diet; mitochondria from rats fed a (●) tocopherol deficient diet, (▲) 10.5 mg α-tocopherol per kilogram of diet; microsomes from rats fed (○) chow diet; sarcosomes from hearts of rats fed (■) tocopherol deficient diet.

TABLE I

Molar Ratios of Oxygen Absorbed Per Fluorescent Compound Produced[a]

Subcellular particle	Dietary α-tocopherol[b]	Hours of peroxidation		
		1	2	4
Mitochondria	0	363	411	473
Mitochondria	10.5	386	275	360
Mitochondria	45	0	0	0
Microsomes	10.5	317	362	408
Microsomes	45	458	436	470
Microsomes	66[c]	1072	912	992

[a]Standard used was the 1-amino-3-iminopropene derivative of glycine.

[b]Milligrams per kilogram of diet containing 10% stripped lard and 1% cod liver oil.

[c]Purina rat chow diet.

studies there was a direct relationship between oxygen absorption and fluorescence development (Fig. 3). It is apparent however that the lipid content of the diet and the level of dietary antioxidant are factors that influence the extent of peroxidation and this relationship. Note that the relationship was similar for all peroxidizing particles from animals fed the higher fat containing diet regardless of tocopherol content. Microsomes from animals fed the lower lipid and higher tocopherol diet (chow diet) absorbed more oxygen per fluorescent compound produced at all time periods than those from animals fed the higher PUFA diets. The age difference between groups one, two and three and group four is another possible factor that could influence the difference in ratio of oxygen absorbed to fluorescence produced.

For comparative purposes measurements of relative fluorescence give adequate information. For quantitative calculations of the relationship between oxygen absorption and fluorescence development a Schiff-base standard was prepared by reaction of glycine with malonaldehyde to form a fluorescent N,N'-disubstituted 1-amino-3-iminopropene (7). The compound has a molar fluorescence one fourth that of quinine sulfate. Under the instrument parameters used in these studies 1 nmole of the standard glycine derivative per milliliter of solvent had 11.6 fluorescence units at a 0.3 meter multiplier setting. The molar ratios of oxygen absorbed per fluorescent compound produced per milligram of protein after 1,2 and 4 hr of peroxidation are shown in Table I. This Table again shows the similarities of the ratios obtained for particles from animals on diets with the same lipid content. The Table does not include values for heart sarcosomes since oxygen uptake was initiated slowly; however the average molar ratio by 8 hr of peroxidation was 240:1.

Production of TBA-Reacting Products

Another parameter used as a measurement of lipid peroxidation is the formation of TBA reacting materials, primarily carbonyl compounds. Table II presents the data obtained for various peroxidizing systems over a 6 hr time course. As has been noted in some reports the production of TBA reactants increased with time during the initial phase of peroxidation and then in microsomal fractions the level of production either stabilized or decreased. The best correlation for the rate of production of TBA reactants with the other indices of peroxidation was found after 1 hr of peroxidation of mitochondria. Oxygen absorption, TBA reactants and fluorescence correlated inversely with the three levels of dietary α-tocopherol.

Effect of Peroxidation on Enzyme Activities

During the course of lipid peroxidation the activities of mitochondrial succinoxidase and microsomal DPNH cytochrome c reductase were measured. The decreases in activities over those of 0-time samples by 2 hr ranged from 35% to 60% and 9% to 45% for succinoxidase and DPNH cytochrome c reductase, respectively; after 6 hr of peroxidation the activities had decreased 83% to 86% and 36% to 74%, respectively.

DISCUSSION

It has long been known that mitochondria isolated from tocopherol deficient animals peroxidize more rapidly than those from animals on diets supplemented with α-tocopherol (2); therefore a correlation between absorption of oxygen by membranous subcellular particles and the tocopherol content of the diets was expected. As found in previous studies (2) of peroxidation, mitochondrial succinoxidase activity decreased with time as did the activity of microsomal DPNH cytochrome c reductase.

TABLE II

Production of TBA Reacting Material During Peroxidation of Mitochondria, Microsomes and Sarcosomes

α-Tocopherol content of diet	Peroxidation time	TBA reactants[a]		
		Mitochondria	Microsomes	Sarcosomes
Tocopherol deficient basal diet[b]	0	0	1.4	0
	0.5	4.5	18.8	—
	1.0	6.5	29.5	1.4
	1.5	7.2	33.5	—
	2.0	7.7	40.2	2.4
	4.0	8.7	40.2	5.3
	6.0	12.3	34.7	6.4
	8.0	—	—	7.2
10.5 mg α-Tocopherol/kg basal diet	0	0	1.5	
	0.5	1.3	21.3	
	1.0	2.9	22.8	
	1.5	5.6	24.4	
	2.0	6.7	26.8	
	4.0	8.9	27.1	
	6.0	12.9	25.4	
45 mg α-Tocopherol/kg basal diet	0	0	0.5	
	0.5	0	8.3	
	1.0	0	14.8	
	1.5	0	17.9	
	2.0	0	18.5	
	4.0	0	19.7	
	6.0	0	19.2	
Chow diet, 66 mg α-Tocopherol/kg diet	0		0.7	
	0.5		15.2	
	1.0		24.8	
	1.5		28.1	
	2.0		34.1	
	4.0		34.9	
	6.0		10.7	

[a]Measured as nmoles of malonaldehyde per milligram of protein.

[b]The tocopherol deficient basal diet is described in the text.

The most interesting and potentially useful data were those showing a direct relationship between absorption of oxygen and production of fluorescent compounds. These two parameters were influenced by both dietary fat composition and dietary α-tocopherol levels. The basal tocopherol deficient diet contained approximately 11% of the total fat as linoleic acid and 4% as higher PUFA. This diet compares with the average American dietary which has similar ratios of PUFA, namely, 15% of the total fat is linoleic acid and 2% is other PUFA. In the chow diet 30% of the fat was linoleic acid and 3% was linolenic acid. The protection afforded the PUFA by dietary antioxidant is shown by the inverse relationship between fluorescence development and the level of α-tocopherol in the diet. In systems where oxygen was absorbed the lowest molar ratio of fluorescent compound produced per oxygen absorbed was for microsomes from animals fed the low fat chow diet that contained 66 mg of α-tocopherol per kilogram of diet. This is probably related to the smaller amounts of highly unsaturated lipids in this diet, which upon peroxidation would produce correspondingly lower levels of conjugated carbonyls and malonaldehyde, precursors of the fluorescent products. Although not shown in the results the initial fluorescence of microsomal fractions from animals fed the basal tocopherol deficient diet also correlated with the dietary tocopherol levels. At zero time the relative fluorescence values and the dietary tocopherol levels per kilogram of diet were: 5.5, 45 mg; 10.9, 10.5 mg; and 18.5, 0 mg. The corresponding value for microsomes from animals fed the chow diet, containing 66 mg of α-tocopherol per kilogram of diet, was 9.0. These values indicate that fluorescence measurements may be useful in studying the in vivo peroxidative status of an animal.

Although it has limitations one of the most useful methods for the determination of lipid peroxidation in tissue homogenates has been the TBA estimation of malonaldehyde and other peroxidation products such as 2,4-dienals or 2-enals. TBA values can increase out of

proportion to oxygen absorption in the presence of ascorbate and it is likely that partially oxidized fatty acids in a tissue homogenate could break down without further oxygen absorption to give additional TBA reacting products (22). In the studies reported here the level of TBA reactants formed in peroxidizing microsomes stabilized or decreased after a period of time (Table I) while oxygen absorption continued (Fig. 2). The decreased values may possibly represent further reactions of malonaldehyde among which a minor reaction is with amines to form fluorescent products. Schiff base products with similar fluorescence characteristics are known to form by reaction of lipid peroxidation products, mainly malonaldehyde and other α-dicarbonyls, with the primary amino groups of ethanolamine, amino acids and nucleic acids (24). These are the fluorescent chromophoric products that were measured and correlated with oxygen absorption and TBA reactions in the studies reported here.

One advantage to using the fluorescence method is the high sensitivity of the technique. The inherent sensitivity of the fluorescence technique allows the measurement of the Schiff base product at a level of one part per billion. On a molar basis the measurement of fluorescence was 10 to 100 times more sensitive than the colorimetric TBA assay used in this study. However the molecular extinction coefficient of 1.56×10^5 also allows measurement of malonaldehyde at a concentration of approximately 0.1 nmole/ml. A second advantage to the fluorescence technique is that fluorescence development closely parallels absorption of oxygen even in the later stages of peroxidation. A third advantage is that the fluorescence technique can be applied to studies of lipid peroxidation in vivo.

In animal tissues, particularly brain, heart and testes (10), fluorescent pigments accumulate with age; their accumulation is also a function of antioxidant nutrition or oxidant damage. These lipofuscin pigments have been characterized histologically and biochemically as complexes of lipid protein substances whose composition and characteristics indicate that they are derived by lipid peroxidation of polyunsaturated lipids of subcellular membranes (25-27) such as those used in the studies reported in this paper. Other studies (10) have shown that chloroform-methanol extracts of fluorescent molecular damage to subcellular membranous organelles have spectral characteristics identical with those of isolated age pigments (430-470 nm maxima for fluorescence and 350-380 nm maxima for excitation); therefore a more direct link of lipid peroxidation deteriorative reactions with formation of fluorescent compounds in animals is provided. This link allows for the quantitative assessment of accumulated damage.

To date there has been no method available to easily quantitate lipid peroxidation damage that occurs in vivo. The methods used most frequently to study lipofuscin pigments are histological techniques especially those employing fluorescence microscopy (28,29). From information gained in the studies reported here interesting and pertinent calculations were made of the magnitude of damage that could occur in vivo. Because of the influence of the type and amount of dietary PUFA and of the variability of antioxidant status of animals only a rough estimation of free radical damage can be made. In spite of the approximate nature of such calculations this type of estimation is the closest that can be made with the techniques and knowledge available. For making these calculations the fluorescent standard used was the 1-amino-3-iminopropene derivative of glycine. On a dry weight basis it has been found (Fletcher, Tappel and Siakotos, unpublished) that 1 mg of human heart age pigment has chloroform-methanol extractable fluorescence equivalent to 0.11 nmole of the 1-amino-3-iminopropene derivative of glycine. Strehler et al. (28) have reported that the mean value for volume of age pigment in human heart is 1.43% (50 year value). For each gram of heart muscle 14.3 mg of age pigment would have accumulated. The fluorescence could thus be equivalent to 1.59 nmoles of fluorescent pigment. Based upon the data shown in Table I for molar ratios of oxygen absorbed per fluorescent compound produced, an average value for these molar ratios for subcellular particles from rats fed diets with PUFA ratios similar to those of the human diet was 393:1. Assuming that in human tissue there would be a similar molar ratio, approximately 625 nmoles of oxygen would have been absorbed with the production of a similar molar amount of free radicals for the fluorescent pigment accumulated in each gram of human heart tissue by age 50 years. In vivo and in vitro free radical damage has been most extensively studied in radiation biology and biochemistry (30). Large magnitude biological effects are produced from small amounts of radiation-induced free radicals. Although less well known and apparently less damaging than those of radiation, the free radical intermediates of lipid peroxidation have been related to a wide variety of biological damage (10,11). Thus the available evidence indicates significant damaging effects from free radical reactions of

0.6 μmole/g tissue. This calculated value is clearly an approximation. Not all fluorescent damaged material produced would accumulate over this time period and the diets of individuals would vary in PUFA and in antioxidant content. The range of average values for moles of oxygen absorbed per mole of fluorescent compound produced during peroxidation of subcellular particles from animals fed various dietary levels of fats and α-tocopherol was approximately 250 to 1,000, which is only a fourfold difference between the lowest and highest values. The lower value is for heart sarcosomes from animals on a tocopherol deficient diet for 14 weeks. One would expect this diet to produce more fluorescence because of the fairly high PUFA content and the lower level of protection afforded the PUFA in the membranes.

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Net Esterification in Vitro of Plasma Cholesterol in Human Primary Hyperlipidemia

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ABSTRACT

Net esterification of cholesterol was studied in vitro in the plasma of normal subjects and of patients with different types of hyperlipidemia. Net esterification of cholesterol was found to be significantly enhanced in types IIa, IV and V of human primary hyperlipidemia. In the plasma of all normal and hyperlipidemic subjects taken as a group, net esterification of cholesterol is significantly correlated with free cholesterol ($p<0.001$) and with phosphatidyl choline ($p<0.001$).

INTRODUCTION

Plasma cholesterol esterification is known to be catalyzed by lecithin-cholesterol acyltransferase (LCAT) (1). It has been reported that experimental hypercholesterolemia in the rabbit, chicken and rat caused a decrease in LCAT activity (2). However, conflicting evidence of enhanced cholesterol esterification in plasma with increased concentration of free cholesterol was found in normal human subjects and in patients with myocardial infarction and familial hypercholesterolemia (3).

The heterogeneity of human primary hyperlipidemia has been clearly established (4). Each of the five types of hyperlipidemia recognized is characterized mainly by an increased concentration of one or two of the lipoprotein classes or by the presence of an abnormal lipoprotein in the plasma. Because LCAT is more active with high density lipoproteins (HDL) than with low density lipoproteins (LDL) (5), and does not appear to act directly on very low density lipoproteins (VLDL) (6), it is likely that an imbalance in the distribution of lipoproteins will influence cholesterol esterification in hyperlipidemic plasma. In the present communication we have studied cholesterol esterification in the plasma of 7 normal subjects and 25 hyperlipidemic subjects with different types of primary hyperlipidemia.

EXPERIMENTAL PROCEDURES

Patient Screening and Analytical Methods

The criteria for the classification of the patients (4) were based on the clinical features, the plasma lipid values and especially the plasma lipoprotein pattern on paper electrophoresis (7). Patients with type II hyperlipidemia were further separated into two subgroups on the basis of plasma triglyceride-cholesterol ratio. Patients with a ratio <0.40 were classified as "type IIa," and those with a ratio ≥ 0.40 as "type IIb." Plasma total cholesterol (8), free cholesterol (9) and triglycerides (10) were measured with an autoanalyzer, and esterified cholesterol was calculated by the difference of total to free cholesterol.

Plasma lipids were extracted (11) and the phospholipids were separated into classes by thin layer chromatography with the solvent system chloroform-methanol-water (65:25:4 v/v/v). Phosphatidyl choline concentration was measured by determination of inorganic phosphorus (12).

Assays for Net Esterification of Plasma Cholesterol

Two different assays were used. In the first method, 0.5 ml of plasma was thoroughly mixed at 4 C with 25 μl of a suspension of 4-^{14}C-cholesterol in human albumin (13) (2 μc/ml) and the mixture was incubated for 24 hr at 37 C. In the second method, a labeled substrate was prepared by incubation for 2 hr at 37 C of a heat-inactivated plasma (14) with a suspension of 4-^{14}C-cholesterol in human albumin (13) containing puromycin (2 mg/ml) and chloramphenicol (4 mg/ml). This labeled plasma substrate was kept frozen at -30 C for periods up to three months. Aliquots (0.8 ml, 350 μg free cholesterol) of the labeled inactivated plasma were incubated at 37 C for 24 hr with 0.2 ml of the plasma to be studied. In each method the reaction was stopped by addition of methanol, and the lipids were immediately extracted (11).

The lipid extract was chromatographed on glass fiber sheets impregnated with silicic acid (Gelman Inst. Co., USA) with the solvent system hexane-diethyl ether-acetic acid (90:10:1 v/v). The fractions identified as cholesterol and cholesteryl esters were cut, dropped into scintillation vials and counted for radioactivity with Permablend II (Packard Instrument Corp., USA) in toluene solution. The yield of the reaction in a microgram of choles-

TABLE I

Plasma Cholesterol Levels and Net Esterification of Cholesterol in Different Types of Hyperlipidemia

		Types of hyperlipidemia				
	Control	IIa[a]	IIb[a]	III	IV	V
n	7	12	4	1	4	4
Total cholesterol, mg/100 ml	169 ± 33[b]	353 ± 81	261 ± 31	560	371 ± 153	246 ± 62
Free cholesterol, mg/100 mg	54 ± 13	130 ± 34	93 ± 12	201	156 ± 97	99 ± 21
Plasma cholesteryl esters, %[c]	68 ± 2	64 ± 3	64 ± 3	64	60 ± 7	58 ± 11
		(NS)[d]	(NS)		($p<0.05$)	($p<0.01$)
Phosphatidyl choline, μg phosphorus/ml	48 ± 4	77 ± 13	71 ± 18	---	118 ± 64	80 ± 14
Net esterification, method I, μg/24hr/ml	224 ± 40	312 ± 78	277 ± 76	207	500 ± 129	359 ± 100
		($p<0.05$)	(NS)		($p<0.001$)	($p<0.05$)
Net esterification, method II, μg/24hr/ml	571 ± 64	654 ± 66	542 ± 120	792	1040 ± 180	794 ± 124
		(NS)	(NS)		($p<0.001$)	($p<0.001$)

[a]Type II was subdivided on the basis of the plasma triglyceride to cholesterol ratio in IIa (<0.40) and IIb (≥ 0.40).

[b]Standard deviation.

[c]Per cent of cholesteryl esters present in plasma and calculated from the values of free and total cholesterol.

[d]Two tailed analyses of variance: comparison of the values obtained in the different types of hyperlipidemia to that in control group ($\alpha=0.05$).

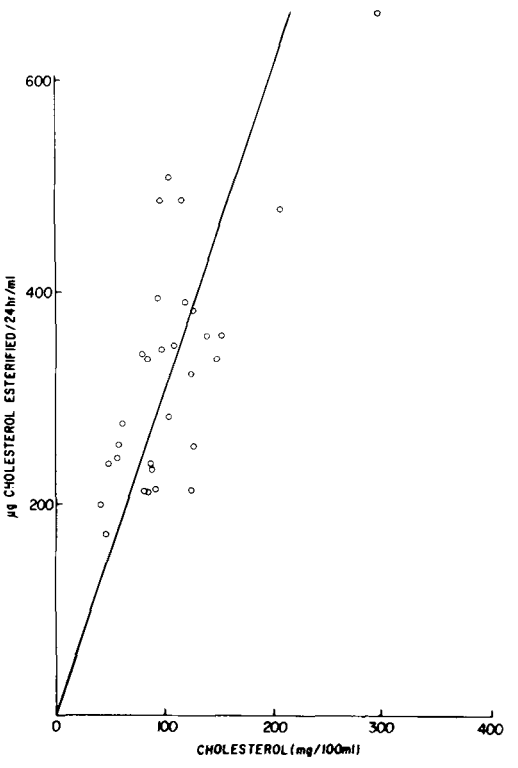

FIG. 1. Correlation between free cholesterol and net esterification (first method) in all normal and hyperlipidemic subjects taken as a group (r=0.731, p<0.001).

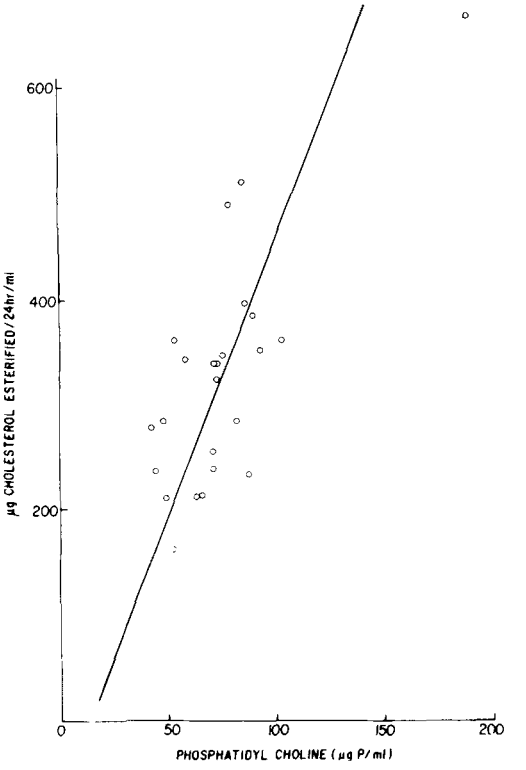

FIG. 2. Correlation between phosphatidyl choline and net esterification (first method) in all normal and hyperlipidemic subjects taken as a group (r=0.768, p<0.001).

terol esterified per 24 hr/ml of active plasma was calculated from the per cent of radioactivity incorporated in cholesteryl esters and the initial concentration of free cholesterol.

In order to demonstrate that the yield of reactions calculated in that manner was representative of the net esterification of endogenous cholesterol, free cholesterol was measured at 0 and 24 hr in the two methods by digitonide precipitation (9). In both methods, net esterification obtained by radioactivity assay was consistently 15% lower than that obtained by colorimetric dosage of cholesterol, results which are in agreement with those found for the initial rate of the reaction (15).

RESULTS AND DISCUSSION

In order to test the reproducibility of the net esterification of plasma cholesterol, blood was withdrawn once a week from four normal subjects over a period of five weeks and each plasma sample was assayed for cholesterol esterification in five separate experiments. The coefficient of variation for each group of five determinations on one plasma sample was found to be 2% by the first method and 8% by the second. In each subject, cholesterol esterification varied from one plasma sample to the other by an average of 11% and 13%, respectively, with each method.

Because in the second method, the ratio of substrate to enzyme concentration (microgram of free cholesterol per milliliter of active plasma) is double that in the first method, the values for net esterification are double those obtained by the first method (Table I). In all types of hyperlipidemia under study, with the exception of type III, both methods give increased yields for net esterification. However the relative increments are more drastic with the first method, probably because of the higher ratio of enzyme to substrate. Since the two different assays are based on the same assumption that exogenous radioactive cholesterol equilibrates with endogenous cholesterol of the lipoproteins, the similarity of the results could be expected. Although the exogenous radioactive cholesterol has been reported to form particulate dispersions whose structure is

different from that of plasma lipoproteins (16), esterification of the radioactive cholesterol was shown to be similar to that of endogenous cholesterol (15,17) and therefore represents a useful tool in the study of plasma cholesterol esterification.

With two tailed analyses of variance the groups were compared in terms of net esterifications (first and second methods) and ratio of esterified to total plasma cholesterol (Table I). As compared to the control group, net esterification (first method) is significantly increased in type IIa ($p<0.05$), type IV ($p<0.001$) and type V ($p<0.05$). The increase in net esterification (second method) is significant only in type IV ($p<0.001$) and type V ($p<0.001$). In spite of the increase in net esterification, the percentage of plasma cholesteryl esters is decreased in hyperlipidemia (Table I). This decrease is statistically significant when all types are taken as a group ($p<0.05$) as well as in type IV ($p<0.05$) and type V ($p<0.01$) taken individually. This apparent contradiction may have a simple explanation. The ratio of esterified cholesterol to total cholesterol decreases together with the density of lipoproteins, i.e., from HDL to LDL to VLDL (18). Therefore, since types V and IV are characterized by an increased concentration of VLDL and type II by an increased concentration of LDL, the ratio of cholesteryl esters to total cholesterol in the unfractionated plasma should be most decreased in types V and IV and less decreased in type II. Indeed, it has been previously reported that patients with essential familial hyperlipidemia have a low percentage of esterified cholesterol in their plasma (19). It is probable that this decrease in plasma cholesteryl esters would be more important if it were not opposed by an increase in net esterification of cholesterol in these types of hyperlipidemia.

In all normal and hyperlipidemic subjects taken as a group, there is a highly significant positive correlation between net esterification (first method) and cholesterol concentration (Fig. 1) and between net esterification (first method) and phosphatidyl choline concentration (Fig. 2). It has been shown (20) that both cholesterol and phosphatidyl choline transfer from VLDL and LDL to HDL, which appears to be the preferred substrate for LCAT. Therefore, whether VLDL or LDL concentrations are increased, both cholesterol and phosphatidyl choline will transfer from these lipoproteins to HDL and be esterified. The highly significant correlations found between substrate concentration and net esterification are supporting evidences for this transfer since LCAT does not react directly with VLDL. These results are in agreement with those of Monger and Nestel (3) who found a positive correlation between LCAT activity and free cholesterol in a group of normal and hypercholesterolemic subjects.

Again in the group which includes all normal and hyperlipidemic subjects, net esterification (second method), is significantly correlated with cholesterol concentration ($r=0.460$, $p<0.02$) and with phosphatidyl choline concentration ($r=0.555$, $p<0.01$). In this method, the fivefold dilution brought about by the inactivated plasma used as substrate minimizes the influence of the lipoproteins in the active plasma studied and decreases the enzyme concentration. This dilution probably accounts for the less significant correlations found with cholesterol and phosphatidyl choline.

In conclusion, we have demonstrated that esterification of plasma cholesterol is significantly increased in types IIa, IV and V of human primary hyperlipidemia. This increase is significantly correlated with the increase of both plasma substrates, cholesterol and phosphatidyl choline. However, since net esterification does not prove whether or not substrate concentrations are rate-limiting, further studies are needed to determine the initial rate of reaction of LCAT in the plasma of normal and hyperlipidemic subjects.

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Mass Spectrometric Analysis of Mono- and Dialkyl Ethers of Diols[1]

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ABSTRACT

Mass spectra of homologous series of long chain mono- and dialkyl ethers of ethanediol and propanediols were measured and general patterns of fragmentation were established. Both classes of diol lipids produce ions which are characteristic for the alkoxy moieties as well as ions which are typical of the constituent short chain diols. Prominent ions are formed by cleavages α and β to the ether oxygen and by rearrangement of one or two hydrogens and concurrent fission. High resolution mass spectrometry and deuterium labeling techniques were used to verify the composition of ions and to substantiate fragmentation mechanisms.

INTRODUCTION

Dialkyl ethers of short chain diols have been shown to occur in the jaw oil of the porpoise *Phocoena phocoena* (2), whereas monoalkyl ethers of short chain diols have not been detected in nature as yet (3). Previous work from our laboratories has shown that mass spectrometry of diol lipids is not only the most useful tool for the detection and analysis of these novel types of lipid constituents (1,4), but has also demonstrated that a better understanding of the mass spectral fragmentation of diol lipids aids in interpreting the more complex spectra of structurally related glycerol-derived lipids (W.J. Baumann et al., in preparation).

Electron impact induced fragmentations of diol ether esters (1) and of simple dialkyl ethers, R-O-R, have been studied previously (5,6). Less is known about the more complex spectra of glycerol ethers (7-9).

In this communication we describe the mass spectra of series of saturated and of some unsaturated monoalkyl ethers and dialkyl ethers of ethanediol and of some of the propanediol derivatives. Compositional assignments of ions and fragmentation mechanisms were verified by high resolution mass spectrometry and specific deuterium labeling.

[1]Part VIII in the series "Naturally Occurring Diol Lipids" (part VII is Reference 1) and part IX in the series "Mass Spectrometry of Lipids" (part VIII is Reference 1).

MATERIALS AND METHODS

Monoalkyl ethers of short chain diols (2-alkoxy-ethanols and 3-alkoxy-propanols) and dialkyl ethers of short chain diols (1,2-dialkoxy-ethanes, 1,2-dialkoxy-propanes (10) and 1,3-dialkoxy-propanes) were prepared as described previously (11). 2-Octadecyloxy-1,1,2,2-tetradeuteroethanol was obtained in 97% isotopic purity by hydrogenation of 2-octadec-1'-enyloxy-1,1,2,2-tetradeuteroethanol (1,12). 1,2-Dioctadecyloxy-1,1,2,2-tetradeuteroethane (isotopic purity of 94%) was prepared by alkylation (11) of the monoalkyl ether. All diol lipids were pure as judged by adsorption chromatography and gas chromatography.

Low resolution mass spectra were recorded on a Hitachi Perkin-Elmer, RMU-6D, single focusing, magnetic scanning instrument at 70 and 15 eV. The samples were introduced through the direct insertion system at a temperature (110-300 C) at which a constant total ion concentration was achieved. Simultaneous scanning of perfluorokerosene which was introduced through the liquid inlet system permitted accurate counting of peaks. Abundances of ions are given as percentages relative to the most prominent peak. Ranges of abundances refer to saturated compounds only. High resolution mass spectra of 2-hexadecyloxy-ethanol and 1,2-dihexadecyloxy-ethane were recorded on an AEI MS-9 instrument at the Molecular Structure Laboratory, Department of Chemistry, Purdue University, Lafayette, Indiana.

RESULTS AND DISCUSSION

The mass spectra of monoalkyl ethers and dialkyl ethers of short chain diols recorded at low electron energies (15 eV) showed the major modes of fragmentation. They were useful in interpreting the more reproducible and more complex spectra measured at 70 eV. Only the latter spectra are discussed. In the spectra of both types of ethers series of peaks are present which are correlated to hydrocarbon ions. The m/e values of the series are: $43+14x,[C_yH_{2y+1}]^+$; $42+14x,[C_yH_{2y}]^+$; $41+14x,[C_yH_{2y-1}]^+$; $40+14x,[C_yH_{2y-2}]^+$; $39+14x,[C_yH_{2y-3}]^+$; $38+14x,[C_yH_{2y-4}]^+$. The composition of these ions was confirmed by precise mass measurements. High resolution

TABLE I

Relative Intensities of Principal Ions in the Mass Spectra of Monoalkyl Ethers of Diols

$(CH_2)_n-OH$ / O-R		n=2		n=2		n=2		n=2		n=2		n=2		n=3		n=3	
		$R=C_{12}H_{25}$		$R=C_{14}H_{29}$		$R=C_{16}H_{33}$		$R=C_{18}H_{37}$		$R=C_{20}H_{41}$		$R=C_{18}H_{35}$[a]		$R=C_{18}H_{37}$		$R=C_{18}H_{35}$[a]	
Ion	Ion structure	m/e	RI	m/e	RI	m/e	RI	m/e	RI	m/e	RI	m/e	RI	m/e	RI	m/e	RI
VII	CH_2OH	31	5.5		5.1		2.9		3.9		3.0		4.7		22		26
	C_3H_7	43	78		79		67		80		80		56		66		60
X	$HO(CH_2)_n$-1	44	4.3		5.4		4.7		5.0		4.0		11	58	20		37
II	$HO(CH_2)_n$	45	49		43		40		39		41		40	59	38		57
	C_4H_9	57	100		100		100		100		100		46		73		69
IV	$HO(CH_2)_nO$	61	0.7		0.8		0.9		0.8		0.6		1.3	75	77		61
XII	$HO(CH_2)_nOH_2$	63	34		42		51		43		57		8.1	77	100		100
	C_5H_{11}	71	58		63		68		64		69		20		67		61
V	$HO(CH_2)_nOCH_2$	75	14		14		18		14		16		6.4	89	81		79
	C_6H_{13}	85	37		39		47		43		49		10		24		21
	C_7H_{15}	99	11		10		14		13		16		2.7		6.1		7.4
VIII	$HO(CH_2)_nO(CH_2)_4$	117	0.8		1.1		2.2		1.3		1.3		1.1	131	0.8		1.3
XI	$R\text{-}C_2H_5$	140	9.0	168	23	196	7.6	224	4.4	252	4.3	222	1.5	224	1.6	222	5.1
IX	R-1	168	6.9	196	9.4	224	9.9	252	4.4	280	5.0	250	8.0	252	2.9	250	25
I	R	169	3.8	197	5.5	225	5.6	253	3.5	281	4.1	251	2.4	253	2.1	251	7.7
III	RO	185	0.4	213	0.6	241	0.6	269	0.5	297	0.4	267	0.5	269	4.5	267	27
VI	$ROCH_2$	199	5.6	227	7.2	255	8.5	283	5.2	311	6.3	281	0.9	283	0.6	281	6.2
	M-18	212	0.1	240	0.2	268	0.2	296	0.1	324	0.1	294	0.1	310	1.1	308	2.2
	M-17	213	0.1	241	0.2	269	0.2	297	0.2	325	0.1	295	0.1	311	4.0	309	0.9
	M	230	0.1	258	0.1	286	0.2	314	0.2	342	0.1	312	0.5	328	3.6	326	7.1

[a]*Cis*-9-octadecenyl.

mass spectrometry also showed that in the series 43+14x ions $[C_yH_{2y-1}O]^+$ and $[C_zH_{2z-3}O_2]^+$ contribute to the peak intensities and that series 42+14x contains $[C_yH_{2y-2}O]^+$. Only the most abundant hydrocarbon ions are listed in the tables I and II, but they are of little diagnostic value.

Monoalkyl Ethers of Diols $R\overset{\alpha}{\cdot}O\overset{\alpha'}{\cdot}(CH_2)_n$-OH

The mass spectra of monoethers of short chain diols show small molecular ion peaks M (0.1-3.6%). Loss of a hydroxy group, $[M-17]^+$, or of water, $[M-18]^+$, does occur and is very pronounced in the case of monoethers of 1,3-propanediol (up to 4.0%).

Cleavage of monoalkyl ethers of ethanediol and 1,3-propanediol in position α to the ether oxygen yields alkyl ions R^+ (I) (2.1-5.6%). Composition of I was confirmed by high resolution mass spectrometry. Ion I is less abundantly produced from unsaturated alkyl moieties (see Table I). α'-Cleavage is more pronounced and is responsible for formation of ion $[HO(CH_2)_n]^+$(II). Both ions, m/e 45 (39-49%) from ethanediol ethers and m/e 59 (38%) from 1,3-propanediol ethers, are approximately equal in intensities. In the spectrum of the monoether of perdeuterated ethanediol ion II (40%) is shifted by four mass units indicating the presence of the intact diol moiety in II. α'-Cleavage and α-cleavage of monoalkyl ethers of ethanediol with charge retention on the oxygen containing fragment produce ions $[RO]^+$ (III) (0.4-0.6%) and $[HO(CH_2)_nO]^+$ (IV) (0.6-0.9%), respectively. Ion IV (1.0%) was shown to contain the intact diol moiety. This type of cleavage was reported to be similarly insignificant for dialkyl ethers, R-O-R (5,6). Monoalkyl ethers of 1,3-propanediol produce ions III and IV in abundances of 4.5% and 77%, respectively.

Cleavage β to the ether bond gives ion $[HO(CH_2)_nOCH_2]^+$ (V), whose composition was confirmed by precise mass measurements. Deuterium labeling of the ethanediol moiety led to a shift of ion V from m/e 75 to m/e 79 (22%), thus confirming its origin. Saturated monoalkyl ethers of ethanediol produce ion V in 14-18% abundance; monoalkyl ethers of propanediol form ion V even more abundantly (81%). Alternative β-cleavages give ions $[ROCH_2]^+$ (VI) and $[HOCH_2]^+$ (VII). Both ions were confirmed by high resolution mass spectrometry and by the increase of two mass units after deuterium labeling of the diol. Abundances of ion VI from monoalkyl ethers of ethanediol are quite independent of chain lengths (5.2-8.5%), unlike what is known of short chain dialkyl ethers (5). Saturated monoalkyl ethers of 1,3-propanediol produce ion VI in much smaller abundance (0.6%). Cleavage β to the alcohol oxygen gives ion $[HOCH_2]^+$ (VII) which is less likely to be formed from monoalkyl ethers of ethanediol (2.9-5.5%) than from the corresponding ethers of 1,3-propanediol (22%).

Under electron impact monoalkyl ethers of diols suffer ϵ-cleavage, leading to ion $[HO(CH_2)_nO(CH_2)_4]^+$ (VIII). Ion VIII which occurs at m/e 117 for ethanediol monoethers (0.8-2.2%) is shifted to m/e 121 (2.3%) for the monoalkyl ether of perdeuterated ethanediol. The elemental composition of VIII was confirmed. In the spectra of the monoalkyl ethers of 1,3-propanediol ion VIII occurs at m/e 131 (0.8%).

Formal loss of the diol moiety from diol monoethers leads to $[R-1]^+$ (IX) as was shown by precise mass measurements. This type of cleavage is more favored for ethanediol monoethers (4.4-9.9%) than it is for monoethers of propanediol. In contrast formal loss of the long chain alcohol from saturated ethanediol monoethers gives ion $[HO(CH_2)_n-1]^+$ X (4.3-5.4%) whereas saturated monoalkyl ethers of propanediol produce ion X in 20% abundance. Unsaturation in the long carbon chain increases the intensities of ions X.

A rather intense ion $[R - C_2H_5]^+$ XI (4.3-23%) is produced primarily from ethanediol monoethers. Ion XI is lower in intensity for 1,3-propanediol ethers and unsaturated analogs (Table I). The mechanism involved in the formation of XI is unknown.

The spectra of monoalkyl ethers of ethanediol and of 1,3-propanediol exhibit a characteristic ion (XII) at one m/e unit higher than expected for the diol, i.e., at m/e 63 for ethanediol ethers and m/e 77 for the propanediol derivatives. In the spectrum of 2-octadecyloxy-1,1,2,2-tetradeuteroethanol ion XII appears at m/e 67 (63%). The intensity of ion XII from ethanediol monoethers increases with increasing chain length of the alkyl group (34-57%). Increasing unsaturation decreases the intensity of this ion. In the spectra of monoalkyl ethers of 1,3-propanediol, ion XII becomes the base peak at m/e 77. A double hydrogen rearrangement may be operative in the formation of ion $[HO(CH_2)_nOH_2]^+$ similar to that postulated in the formation of $[ROH_2]^+$ from dialkyl ethers (6). Hydrogen transfers from the C-4 and C-5 methylene groups appear to be involved in the formation of XII (1,6). The alternative type of ion, $[ROH_2]^+$, was not formed from monoalkyl

TABLE II
Relative Intensities of Principal Ions in the Mass Spectra of Dialkyl Ethers of Diols

		n=2		n=2		n=2		n=2		n=2		n=3		n=3		CH_2-O-$C_{18}H_{37}$	
O-R		R=$C_{16}H_{33}$		R=$C_{18}H_{37}$		R=$C_{16}H_{33}$		R=$C_{18}H_{35}$[a]		R=$C_{18}H_{35}$[a]		R=$C_{18}H_{37}$		R=$C_{18}H_{35}$[a]		CH-O-$C_{18}H_{37}$	
$(CH_2)_n$																CH_3	
O-R'		R'=$C_{16}H_{33}$		R'=$C_{18}H_{37}$		R'=$C_{18}H_{37}$		R'=$C_{18}H_{37}$		R'=$C_{18}H_{35}$[a]		R'=$C_{18}H_{37}$		R'=$C_{18}H_{37}$			
Ion	Ion structure	m/e	RI	m/e	RI	m/e	RI	m/e	RI	m/e	RI	m/e	RI	m/e	RI	m/e	RI
	C_3H_7	43	67		53		61		64		72		51		76		72
	C_4H_9	57	100		100		100		100		77		85		100		100
XII	$HO(CH_2)_nOH_2$	63	31		27		25		21		7.9	77	13		33		1.3
	C_5H_{11}	71	72		75		83		69		40		72		69		72
V	$HO(CH_2)_nOCH_2$	75	6.9		5.4		5.9		3.7		2.8	89	8.0		6.9		1.3
	C_6H_{13}	85	49		59		63		55		25		49		41		52
	C_7H_{15}	99	16		22		2.2		19		7.1		18		13		21
XI	R-C_2H_5	196	7.4	224	6.3	196	7.0	222	6.0	222	3.1	224	4.2	222	1.7	224	0.4
	R'-C_2H_5					224	21	224	1.6					224	1.8		
XIX	R-3	222	1.7	250	8.8	222	7.2	248	2.7	248	2.2	250	15	248	1.5	250	2.1
	R'-3					250	7.0	250	85					250	9.0		
IX	R-1	224	20	252	17	224	21	250	85	250	40	252	15	250	9.0	252	1.3
	R'-1					252	11	252	4.2					252	4.5		
I	R	225	19	253	22	225	20	251	22	251	11	253	5.8	251	3.6	253	28
	R'					253	15	253	12					253	2.2		
III	RO	241	18	269	19	241	15	267	25	267	2.8	269	2.6	267	5.9	269	1.2
	R'O					269	14	269	2.5					269	1.0		
XVII	ROH_2	243	0.8	271	0.9	243	1.2	269	2.5	269	0.9	271	0.3	269	1.0	271	0.3
	$R'OH_2$					271	3.3	271	0.5					271	0.3		
XX	RO+12	253	1.6	281	2.5	253	15	279	1.2	279	1.6	281	100	279	1.8	281	0.9
	R'O+12					281	2.8	281	6.7					281	33		
VI	$ROCH_2$	255	3.1	283	3.5	255	3.8	281	6.7	281	2.9	283	3.6	281	33	283	0.6
	$R'OCH_2$					283	3.2	283	1.5					283	1.6	297	28
XVI	$RO(CH_2)_n$-1	268	0.7	296	0.7	268	1.8	294	1.1	294	0.6	310	31	308	2.5	310	0.2
	$R'O(CH_2)_n$-1					296	1.1	296	1.1					310	11		
XIII	$RO(CH_2)_n$	269	1.0	297	1.3	269	14	295	1.0	295	0.6	311	9.0	309	1.5	311	0.4
	$R'O(CH_2)_n$					297	1.2	297	1.4					311	6.0		
XXI	$RO(CH_2)_n$+2	271	3.0	299	3.4	271	3.3	297	1.4	297	0.5	313	1.2	311	6.0	313	0.2
	$R'O(CH_2)_n$+2					299	5.0	299	0.8					313	0.9		
XIV	$RO(CH_2)_nO$	285	3.9	313	4.0	285	4.3	311	1.9	311	1.4	327	4.7	325	1.5	327	0.5
	$R'O(CH_2)_nO$					313	2.1	313	45					327	4.2		
XVIII	$RO(CH_2)_nOH_2$	287	42	315	38	287	38	313	45	313	5.7	329	12	327	4.2	329	0.4
	$R'O(CH_2)_nOH_2$					315	30	315	23					329	12		
XV	$RO(CH_2)_nOCH_2$	299	2.9	327	3.3	299	5.0	325	7.1	325	1.7	341	7.5	339	1.1	341	0.3
	$R'O(CH_2)_nOCH_2$					327	2.8	327	1.2					341	1.7		
	M	510	0.5	566	0.4	538	1.1	564	9.3	562	5.9	580	1.3	578	30	580	0.2

[a]*Cis*-9-octadecenyl.

ethers of ethanediol and propanediol, thus supporting the above mechanism.

Dialkyl Ethers of Diols $R\overset{\alpha}{\cdot}O\overset{\alpha'}{\cdot}(CH_2)_n\overset{\alpha'}{\cdot}O\overset{\alpha}{\cdot}R'$

The mass spectra of the saturated, long chain dialkyl ethers of ethanediol and propanediols show recognizable molecular ion peaks (0.2-1.3%). Stronger signals are observed for the unsaturated analogs (5.9-30%). Ions which would be formed through loss of a hydroxy group, $[M-17]^+$, or through loss of water, $[M-18]^+$, do not occur.

Cleavage of dialkyl diol ethers at the bonds α to the ether oxygens leads to ions $[R]^+$ and $[R']^+$ (I). α'-Cleavage produces ions $[RO(CH_2)_n]^+$ and $[R'O(CH_2)_n]^+$ (XIII)(Table II). The elemental compositions of ions I and XIII were confirmed by high resolution mass spectrometry. In the spectrum of the diether of perdeuterated ethanediol only ion XIII (0.4%) was shifted upwards by four mass units as expected. Formation of ions I from saturated ethanediol diethers is greatly favored (19-22%) over formation of ions XIII (0.6-1.3%). In the spectra of 1,3-propanediol diethers, ion I is less abundant (5.8%) than ion XIII (9.0%), whereas in the spectra of the 1,2-propanediol derivatives only ion I (28%) is of significance. α-Cleavage of the ether bond with charge retention on the oxygen containing fragment gives ions $[RO(CH_2)_nO]^+$ and $[R'O(CH_2)_nO]^+$ (XIV), α'-cleavage affords ions $[RO]^+$ and $[R'O]^+$ (III). The results of precise mass measurements and of deuterium labeling in the diol moiety agree with these structures. Formation of ions III (18-19%) from saturated ethanediol diethers is favored over formation of XIV (3.9-4.0%). Ion III is produced in lower abundance from diethers of propanediols (1.2-2.6%).

Cleavages β to the ether oxygen in dialkyl ethers of short chain diols afford ions $[ROCH_2]^+$ and $[R'OCH_2]^+$ (VI), and $[RO(CH_2)_nOCH_2]^+$ and $[R'O(CH_2)_nOCH_2]^+$ (XV). Again the composition of these ions was confirmed by high resolution mass spectrometry. The spectrum of the dialkyl ether of perdeuterated ethanediol showed ions VI and XV at two and four mass units greater, respectively. Symmetrical saturated dialkyl ethers of ethanediol produce ions VI (3.1-3.5%) and XV (2.9-3.3%) in similar abundances. In the spectra of the 1,3-propanediol diethers ion XV (7.5%) is slightly more intense than ion VI (3.6%). In the spectra of dialkyl ethers of 1,2-propanediol ion XV is negligible. However β cleavage gives rise to two ions of the type VI, $[ROCH_2]^+$ (0.6%) and $[ROCHCH_3]^+$ (28%).

Formal loss of monoalkyl diol ether from α,ω-alkanediol diether, i.e., α-fragmentation of the ether bond with concurrent rearrangement of one hydrogen atom, leads to the formation of olefinic ions $[R-1]^+$ and $[R'-1]^+$ (IX) in high abundance (15-20%). Their elemental composition was confirmed. In the spectrum of 1,2-di-*cis*-9-octadecenyloxy-ethane, ion IX is observed in exceptionally high abundance (40%). Alternatively formal loss of alkanol from ethanediol diethers is less favored yielding ions $[RO(CH_2)_n-1]^+$ and $[R'O(CH_2)_n-1]^+$ (XVI) 0.7%). Peaks for both ions IX and XVI are very intense in the spectra of 1,3-propanediol derivatives (15 and 31%, respectively), but they are weak for 1,2-propanediol diethers (1.3 and 0.2%). When the diol moiety of dioctadecyl ethanediol ether was deuterated ions corresponding to XVI were shifted by 3 or 4 mass units suggesting loss of either one hydrogen or one deuterium. The structure of these ions remains to be elucidated.

Cleavages of the ether bond in dialkyl ethers of diols and concurrent rearrangement of two hydrogens either afford $[ROH_2]^+$ and $[R'OH_2]^+$ (XVII) or $[RO(CH_2)_nOH_2]^+$ and $[R'O(CH_2)_nOH_2]^+$ (XVIII). The elemental compositions of these ions were confirmed. In the spectrum of the dialkyl ether of perdeuterated ethanediol ion XVIII was shifted by four mass units whereas ion XVII did not contain deuterium. The hydrogens transferred probably originate from the C-4 and C-5 methylene groups as mentioned before for monoethers. Formation of ions XVIII is greatly favored over that of XVII. In the spectra of dialkyl ethers of ethanediol, ion XVIII is a major ion (38-42%) whereas ion XVII is of little significance (0.8-0.9%). 1,3-Propanediol diethers produce ion XVIII in about 12% abundance but ion XVII is absent because its formation would required hydrogen transfer from a position occupied by the ether oxygen. Neither of these ions is formed from 1,2-propanediol diethers. Doubly protonated "alkoxy" ions have been described as unstable and presumably lose water quite readily (6). Hence ions XVII and XVIII could be expected to form ions I and XIII, respectively. However metastable peaks were not observed for such transitions.

The mass spectra of dialkyl ethers exhibit ions $[R-3]^+$ and $[R'-3]^+$ (XIX) whose elemental compositions were confirmed. Dialkyl ethers of ethanediol produce ions XIX in medium abundance (7.0-8.8%). Formation of XIX from 1,3-propanediol diethers is more pronounced (15%) but is less significant from 1,2-propandiol diethers (2.1%). The same ion was observed previously in the spectra of alkyl ether esters of diols (1), alk-1-enyl ether esters

of ethanediol (1) and to a small extent in the spectra of dialkyl ethers (6). The mechanism leading to ion XIX remains unexplained.

Similarly the structure and mode of formation of another ion (XX) which is formed from dialkyl ethers of diols and which formally contains the alkoxy residue plus one carbon atom remains to be elucidated. Precise mass measurements confirmed the composition of XX as $C_{m+1}H_{2m+1}O$. The spectrum of the dialkyl ether of perdeuterated ethanediol suggested some scrambling. Ion XX is formed in 1.6-2.5% abundance from dialkyl ethers of ethanediol and in 0.9% abundance from 1,2-propanediol derivatives. However in the spectrum of 1,3-dioctadecyloxy-propane ion XX gives rise to the base peak at m/e 281. We have previously shown (1) that the same ion is produced from alkyl ether esters of diols in relatively high abundance (13-21%). Ions of the type $[RO(CH_2)_n+2]^+$ (XXI) (3.0-3.4%) are formed from dialkyl ethers of ethanediol only. Their elemental composition, $C_{m+2}H_{2m+6}$, was verified by precise mass measurements. Deuteration in the diol moiety shifted ion XXI by four mass units showing that all deuterium atoms were retained. Dialkyl ethers of ethanediol and 1,3-propanediol also produce hydrocarbon ions $[R - C_2H_5]^+$ and $[R' - C_2H_5]^+$ (XI) (4.2-7.4%). Ion XI is formed in lower abundance (0.4%) from 1,2-propanediol diethers.

Some low mass ions merit comment since they contain the diol moiety and thus aid in differentiating dialkyl ethers derived from different diols. These ions of the structure $[HO(CH_2)_nOH_2]^+$ (XII) occur in an abundance of 27-31% in the mass spectra of dialkyl ethers of ethanediol (m/e 63) and are shifted by four mass units upon deuteration of the ethanediol moiety. Similar abundances are found for 1,3-propanediol derivatives. The mechanism of formation of ion XII probably involves two rearrangements, i.e., single and double hydrogen transfers. In the spectrum of 1,2-dioctadecyloxy-propane the corresponding "diol ion" $[HOCH_2CH(CH_3)OH_2]^+$, occurs at much lower abundance (1.3%). Generally in dialkyl ethers of 1,2-propanediol, rearrangements appear to be less favored than fragmentations. This may be partially due to enhanced β-cleavage next to the methyl branch, partially to steric hindrance of hydrogen transfers.

The mass spectra of diethers of ethanediol and 1,3-propanediol also show significant ions at m/e 75 (5.4-8.0%) and m/e 89 (8.0%), respectively. High resolution spectra and deuterium labeling suggested the structure $[HO(CH_2)_nOCH_2]^+$ (V) for these ions.

DISCUSSION

Mass spectra of mono- and dialkyl ethers of ethanediol and propanediols exhibit patterns of fragmentation that are characteristic for each of the individual compounds. Furthermore differences exist between the mass spectra of mono- and dialkyl ethers as well as between these diol lipids derived from different diols.

Monoalkyl ethers of short chain diols preferentially undergo α-cleavage with concurrent double hydrogen rearrangement to give ion XII, α'-cleavage (II) or β-cleavage (V). Formal loss of alcohol (IX and X), α-cleavage (I), α-cleavage and loss of a C_2H_5 group (XI) or β-cleavage (VI) occur to a lesser degree.

Dialkyl ethers of 1,2-ethanediol undergo rearrangements rather than cleavages with the exception of α- and α'-cleavages giving ions I and III, respectively. Major modes of ion formation involve α-cleavage with concurrent rearrangement of two hydrogens (XVIII), a complicated rearrangement resulting in the formation of a protonated ethanediol (XII), and the formal elimination of 2-alkoxy-ethanol (IX). Less predominant ions are produced by α-cleavage and expulsion of C_2H_5 (XI), β-cleavage (V), and by an unknown mechanism leading to ion XIX. Dialkyl ethers of 1,3-propanediol undergo similar rearrangements. In addition formal loss of long chain alcohol (XVI) and the unexplained rearrangement giving rise to the base peak (XX) do occur with 1,3-propanediol diethers. In contrast to both dialkyl ethers of ethanediol and of 1,3-propanediol, diethers of 1,2-propanediol predominantly suffer α-cleavages (I) and β-cleavages (VI) whereas rearrangements play a secondary role.

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Synthesis of C-18 Mixed Acid Diacyl-*sn*-Glycerol Enantiomers[1]

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ABSTRACT

Procedures have been developed for the synthesis of both enantiomeric forms of mixed fatty acid, saturated and polyunsaturated 1,2-diacyl-*sn*-glycerols and 2,3-diacyl-*sn*-glycerols from D-mannitol as starting material. The following diacyl-*sn*-glycerols have been synthesized: 1-Stearoyl-2-linoleoyl-*sn*-glycerol, 1-stearoyl-2-linolenoyl-*sn*-glycerol, 2-linoleoyl-3-stearoyl-*sn*-glycerol and 2-linolenoyl-3-oleoyl-*sn*-glycerol. Their specific rotations, refractive indices, densities, solubilities, carbon and hydrogen analysis and iodine values have been reported.

INTRODUCTION

In order to obtain reference compounds for the natural diacyl-*sn*-glycerols when isolated, it has been necessary to devise methods of synthesis of various types of diacyl-*sn*-glycerols to duplicate the natural products.

The synthesis of optically active 1,2-diacyl-*sn*-glycerols has been attempted by many investigators through the optical resolution of the racemic intermediates possessing acidic or basic groups as precursors of the desired optically active acyl-*sn*-glycerols (1-5). However none of these methods has yielded enantiomerically pure diacyl-*sn*-glycerols. The synthesis of enantiomerically pure 1,2-distearoyl-*sn*-glycerol, 1,2-dipalmitoyl-*sn*-glycerol and 1,2-dimyristoyl-*sn*-glycerol was achieved by Sowden and Fischer in 1941 from 1,2-isopropylidene-*sn*-glycerol (6). This was obtained from naturally occurring D-mannitol by the methods that transfer the asymmetry of the carbon 2 and 5 of D-mannitol to the 1,2-isopropylidene-*sn*-glycerol (7-9). Thus the 1,2-isopropylidene-*sn*-glycerol provides the stereochemical key substance as starting material for the synthesis of optically active 1,2-diacyl-*sn*-glycerols. Howe and Malkin (10) used the procedure of Sowden and Fischer for the synthesis of the corresponding racemic compounds and they were able to improve the method, obtaining higher yields in each stage of synthesis.

All such diacyl-*sn*-glycerols prepared to that date contained solely saturated fatty acid substituents, while the unsaturated acid 1,2-diacyl-*sn*-glycerols had defied attempts at synthesis.

In 1958 Baer and Buchnea (11) reported for the first time the synthesis of 1,2-dioleoyl-*sn*-glycerol and 2,3-dioleoyl-*sn*-glycerol by converting the oleic acid substituents into 9,10-dibromostearic acids and regenerating the double bond with activated zinc after removal of the protective benzyl group from the 3 position by catalytic hydrogenolysis. Two years later Buchnea and Baer (12) reported the synthesis of 1-stearoyl-2-oleoyl-*sn*-glycerol and 1-oleoyl-2-stearoyl-*sn*-glycerol. These methods are readily adapted to the synthesis of monounsaturated acid diacyl-*sn*-glycerols and mixed acid, saturated and monounsaturated diacyl-*sn*-glycerols, but not of course to the synthesis of mixed acid saturated and polyunsaturated, diacyl-*sn*-glycerols.

Recently, Pfeiffer et al. (13) were able to synthesize optically active, polyunsaturated 1,2-diacyl-*sn*-glycerols by employing 2,2,2-trichloroethoxycarbonyl as a protecting group. So

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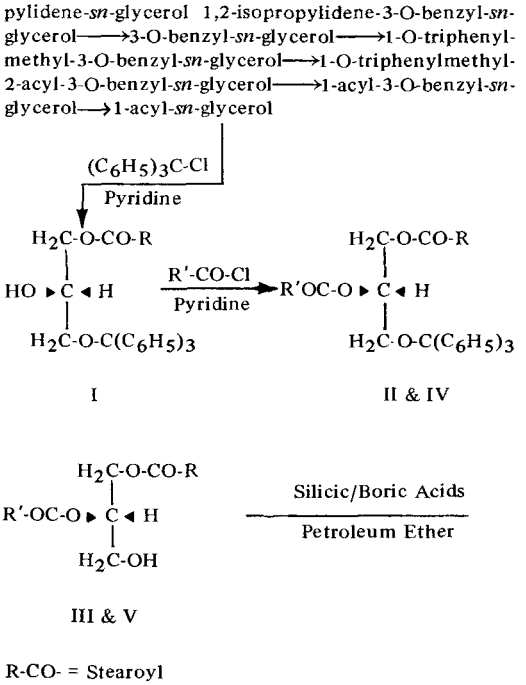


FIG. 1. Synthesis of 1,2-diacyl-*sn*-glycerol from D-mannitol via above intermediates.

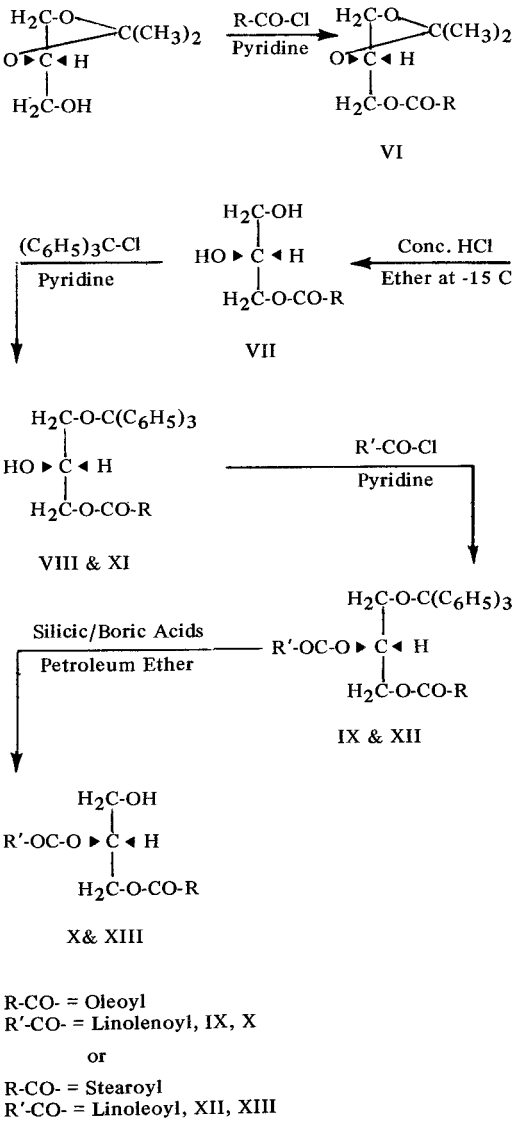
far to the author's knowledge, no enantiomeric, mixed acid, saturated and polyunsaturated, 1,2-diacyl-*sn*-glycerols and 2,3-diacyl-*sn*-glycerols have been synthesized.

Now a general procedure is reported that permits the synthesis of mixed acid, saturated and polyunsaturated, 1,2-diacyl-*sn*-glycerols and 2,3-diacyl-*sn*-glycerols using D-mannitol as a starting material.

The synthesis of 1-stearoyl-2-linoleoyl-*sn*-glycerol, 1-stearoyl-2-linolenoyl-*sn*-glycerol starts with 1-stearoyl-*sn*-glycerol, and the synthesis of 2-linoleoyl-3-stearoyl-*sn*-glycerol and 2-linolenoyl-3-oleoyl-*sn*-glycerol starts with 3-stearoyl-*sn*-glycerol and 3-oleoyl-*sn*-glycerol, respectively.

1-Stearoyl-*sn*-glycerol was obtained by two different synthetic pathways: (a) from D-mannitol by a more complicated procedure (12) as in Figure 1, and (b) by a relatively simple procedure using the same sequence of reactions as illustrated in Figure 2, but starting from L-mannitol (14).

The synthesis of 1-stearoyl-2-linoleoyl-*sn*-glycerol and 1-stearoyl-2-linolenoyl-*sn*-glycerol is illustrated in the reaction scheme, Figure 1, and the synthesis of 2-linoleoyl-3-stearoyl-*sn*-glycerol and 2-linolenoyl-3-oleoyl-*sn*-glycerol is illustrated in the reaction scheme, Figure 2.



R-CO- = Oleoyl
R'-CO- = Linolenoyl, IX, X

or

R-CO- = Stearoyl
R'-CO- = Linoleoyl, XII, XIII

FIG. 2. Synthesis of 2,3-diacyl-*sn*-glycerol from D-mannitol via above intermediates.

EXPERIMENTAL PROCEDURES

Materials

L-mannitol was prepared from quebracitol via L-inositol. (The method for the preparation of L-mannitol (15,16,17) has been simplified and adapted to the preparation of larger amounts. The details of preparation will be reported elsewhere.) D-mannitol (certified) was obtained from Fisher Scientific Company. Triphenylmethyl chloride was prepared by methods of Gomberg (18,19) as described by Bachmann (20). For further purification the triphenylmethyl chloride was distilled in vacuo, bp 155 C/0.08 mm Hg; mp 113-114 C. Linoleic acid and linolenic acid of high purity were obtained from the Hormel Institute, University of Minnesota. Oleic acid of a purity of at least 99.8% was prepared according to the method of Rubin and Paisley (21). Stearic acid, 99.5% pure, was obtained from Fluka A.G. Buchs SG, Switzerland. All fatty acids were converted into the corresponding chlorides with oxalyl chloride. Anhydrous pyridine was prepared from "Certified," infrared analyzed pyridine. The benzene was thiophene free and dried over sodium wire. The silicic acid was Mallinckrodt, 100 mesh (powder), "Analytical Reagent" with a 12% loss in weight on ignition. Boric acid was certified Fisher Scientific Company Stock. All experiments with unsaturated fatty acids were carried out under a nitrogen atmosphere.

Preparation of 1-Stearoyl-3-O-Triphenylmethyl-*sn*-Glycerol (I)

1-Stearoyl-*sn*-glycerol (25.0 g, 0.07 mole) was dissolved in 200 ml of a mixture of anhydrous pyridine and anhydrous benzene (1:1 v/v). A solution of 19.5 g (0.07 mole) of pure triphenylmethyl chloride in 50 ml of anhydrous benzene was added with stirring under anhydrous conditions. The reaction mix-

TABLE I

Analytical Data and Yields of the Acyl-Isopropylidene-*sn*-Glycerol (VI), Monoacyl-*sn*-Glycerol (VII), Monoacyl-Monotriphenylmethyl-*sn*-Glycerols (I, VIII, IX), Diacyl-Monotriphenylmethyl-*sn*-Glycerols (II, IV, IX, XII) and Diacyl-*sn*-Glycerols (III, V, X, XIII)

Compounds	Formula and mol wt	Per cent carbon		Per cent hydrogen		Iodine value		Per cent yield
		calc.	found	calc.	found	calc.	found	
I	$C_{40}H_{56}O_4$ (601)	79.95	79.99	9.40	9.47	---	---	73
II	$C_{58}H_{87}O_5$ (864)	80.60	81.00	10.14	10.21	58.7	59.0	95
III	$C_{39}H_{72}O_5$ (621)	75.42	75.33	11.70	11.57	81.8	82.1	95
IV	$C_{58}H_{85}O_5$ (862)	80.78	80.91	9.93	9.89	88.3	88.7	95
V	$C_{39}H_{70}O_5$ (619)	75.67	75.55	11.40	11.42	123.0	122.5	76
VI	$C_{24}H_{44}O_4$ (296)	72.68	72.62	11.20	11.16	64.0	63.8	96
VII	$C_{21}H_{40}O_4$ (357)	70.08	69.98	11.87	12.00	71.2	71.2	92
VIII	$C_{40}H_{54}O_4$ (599)	80.20	80.15	9.10	9.23	42.4	42.5	80
IX	$C_{58}H_{83}O_5$ (860)	80.97	81.01	9.72	9.68	118.1	119.0	95
X	$C_{39}H_{68}O_5$ (617)	75.95	76.00	11.12	11.21	164.6	165.0	56
XI	$C_{40}H_{56}O_4$ (601)	79.95	79.92	9.40	9.50	---	---	95
XII	$C_{58}H_{87}O_5$ (864)	80.60	80.35	10.14	10.18	58.7	58.9	93
XIII	$C_{39}H_{72}O_5$ (621)	75.42	75.50	11.70	11.58	81.8	82.0	75

ture was kept at 45 C for 24 hr and then the reaction product, 1-stearoyl-3-O-triphenylmethyl-*sn*-glycerol was isolated and purified as follows: The reaction mixture was diluted with 300 ml of diethyl ether and the mixture was washed in succession with a 300 ml portion of distilled water, two 300 ml portions of ice cold 2 N sulfuric acid, one 300 ml portion of distilled water, two 300 ml portions of a saturated sodium bicarbonate solution, and finally with two 300 ml portions of distilled water. The solution was dried with 100 g of anhydrous sodium sulfate, and the solvents were removed by distillation under reduced pressure from a bath at 35 C. The remaining material was kept in vacuo at 0.08 mm Hg until its weight was constant. The 1-stearoyl-3-O-triphenylmethyl-*sn*-glycerol weighed 41.0 g, and was shown to be a fairly homogeneous substance by thin layer chromatography. Two recrystallizations from petroleum ether (bp 30-60 C) at +6 C yielded a chromatographically pure material.

Preparation of 1-Stearoyl-2-Linoleoyl-3-O-Triphenylmethyl-*sn*-Glycerol (II)

To a solution of freshly prepared 1-stearoyl-3-O-triphenylmethyl-*sn*-glycerol (I), 18.0 g (0.03 mole) in 60 ml of anhydrous benzene and 5 ml of anhydrous pyridine was added freshly prepared and distilled linoleoyl chloride, 9.0 g (0.03 mole) in 20 ml of anhydrous benzene. The reaction mixture was kept under anhydrous conditions at 40 C for 24 hr, and then diluted with 200 ml of diethyl ether. The mixture was washed in succession with a 250 ml portion of distilled water, with two 250 ml portions of ice cold 2 N sulfuric acid, two 250 ml portions of saturated sodium bicarbonate solution and finally two 250 ml portions of distilled water. The solution was dried with 50 g of anhydrous sodium sulfate and the solvents were evaporated under reduced pressure. The remaining oil was dissolved in 200 ml of petroleum ether (bp 30-60 C) and the solution was kept for 20 hr at -6 C. The turbidity was removed by centrifugation. The clear supernatant was decanted and again concentrated under reduced pressure. The concentrated product was kept in vacuo of 0.05 mm Hg until its weight was constant. The 1-stearoyl-2-linoleoyl-3-O-triphenylmethyl-*sn*-glycerol, a viscous oil, weighed 24.5 g.

The material without further purification exhibited a specific rotation of $[\alpha]_D + 12.2°$ in chloroform *c*,10 and an iodine value of 59.0, calculated 58.7. Thin layer chromatography showed a quite homogeneous material.

To obtain analytically pure material, 1-stearoyl-2-linoleoyl-3-O-triphenylmethyl-*sn*-glycerol was chromatographed on silicic acid, although it involves partial detritylation. A solution of 23 g of material dissolved in 100 ml of benzene (U.S.P., redistilled) was passed through a 300 g silicic acid column, 3.5 cm wide and 50 cm long. The column was eluted with benzene until eluate was free of solute. In the initial benzene eluate pure 1-stearoyl-2-linoleoyl-3-O-triphenylmethyl-*sn*-glycerol was recovered corresponding to about 70% of the material applied to the column. In the later benzene eluate triphenylcarbinol appeared in small amounts. The benzene-diethyl ether mixture (4:1 v/v) eluted the detritylated product, 1-stearoyl-2-linoleoyl-*sn*-glycerol (5 g) with about 30% yield.

Preparation of 1-Stearoyl-2-Linoleoyl-*sn*-Glycerol (III)

The removal of the triphenylmethyl protec-

TABLE II

Physical Properties of the Acyl-Isopropylidene-*sn*-Glycerol, Monoacyl-*sn*-Glycerol, Monoacyl-Monotriphenylmethyl-*sn*-Glycerols, Diacyl-Monotriphenylmethyl-*sn*-Glycerols and Diacyl-*sn*-Glycerols

Compounds	Specific rotation in chloroform c,10, deg	Refractive index at 25 C	Density at 20 C	Physical state at 20 C
I	+ 3.85	---	----	Crystalline
II	+12.50	---	---	Oil
III	- 2.80	1.4710	0.9230	Oil
IV	+12.40	1.5125	---	Oil
V	- 2.70	1.4702	0.9315	Oil
VI	+ 4.80	1.4560	0.9109	Oil
VII	-- 3.60[a]	---	---	Paste
VIII	- 3.60	---	---	Oil
IX	12.00	1.5240	---	Oil
X	+ 2.60	1.4800	0.9318	Oil
XI	- 3.70	---	---	Crystalline
XII	-12.30	---	---	Oil
XIII	+ 2.70	1.4710	0.9230	Oil

[a]Specific rotation measured in pyridine c,10. Reported for 3-stearoyl-*sn*-glycerol -3.58 deg, in pyridine c,12.3(14).

tive group on a silicic acid column varied from one diacyl-*sn*-glycerol to another, and also from one batch of silicic acid to the other. However later work showed that silicic acid containing 10% by weight of boric acid offers an excellent mixture for the complete removal of triphenylmethyl protective group from any diacyl-O-triphenylmethyl-*sn*-glycerol.

The 1-stearoyl-2-linoleoyl-3-O-triphenylmethyl-*sn*-glycerol (14 g) was dissolved in 150 ml of petroleum ether (bp 30-60 C) and the solution was passed through a column of 300 g of a freshly prepared silicic acid-boric acid mixture (10:1 w/w). (The experimental preparation and application of silicic acid-boric acid mixture for the removal of triphenylmethyl protective groups is explained in a separate paper, now in preparation.)

The column was 3.5 cm wide and 60 cm long, and was eluted with petroleum ether, petroleum ether-diethyl ether (96:4 v/v) and petroleum ether diethyl ether (90:10 v/v). The petroleum ether fraction eluted only very small amounts of original material. Triphenylcarbonol was eluted with petroleum ether-diethyl ether mixture (96:4 v/v), and the detritylated product, 1-stearoyl-2-linoleoyl-*sn*-glycerol was recovered in the petroleum ether-diethyl ether mixture (90:10 v/v) in about a 95% yield. 1-Stearoyl-2-linoleoyl-*sn*-glycerol is an oil at room temperature and solidified gradually at +6 C.

1-Stearoyl-2-Linolenoyl-3-O-Triphenylmethyl-*sn*-Glycerol (IV), and 1-Stearoyl-2-Linolenoyl-*sn*-Glycerol (V)

These were prepared by the same procedure as II and III with proper choice of acylating reagent.

Preparation of 1,2-Isopropylidene-3-Oleoyl-*sn*-Glycerol (VI)

The method for the synthesis of saturated 3-acyl-*sn*-glycerols by Baer and Fischer (14) was adapted to the synthesis of 3-oleoyl-*sn*-glycerol. To a solution of freshly prepared 1,2-isopropylidene-*sn*-glycerol 6.8 g (0.05 mole) and 5 ml of anhydrous pyridine in 50 ml of anhydrous benzene was added 15.0 g (0.05 mole) of freshly prepared and distilled oleoyl chloride dissolved in 50 ml of anhydrous benzene. The reaction mixture was kept for 24 hr at room temperature, diluted with 150 ml of diethyl ether, and then freed of pyridine with ice cold 1 N sulfuric acid, then with saturated sodium bicarbonate solution, and finally with water. After drying the diethyl ether-benzene layer with anhydrous sodium sulfate the solvents were evaporated under reduced pressure to give 18.8 g of 1,2-isopropylidene-3-oleoyl-*sn*-glycerol.

Preparation of 3-Oleoyl-*sn*-Glycerol (VII)

To a -15 C solution of 1,2-isopropylidene-3-oleoyl-*sn*-glycerol in 100 ml of diethyl ether was added 100 ml of -15 C concentrated hydrochloric acid. The reaction mixture was stirred for 15 min and then diluted with 800 ml of ice cold distilled water. The reaction mixture was allowed to stand with occasional stirring for 20 min in a bath of -15 C ice-salt mixture. The 3-oleoyl-*sn*-glycerol was then extracted three times with 300 ml portions of diethyl ether. The combined diethyl ether extracts after washing with 300 ml of ice cold distilled

water were dried over 100 g of anhydrous sodium sulfate. The solvent was then removed by distillation under reduced pressure at 30 C bath temperature. The remaining material was kept in vacuo at 0.05 Hg until the weight was constant. The 3-oleoyl-*sn*-glycerol (weight 15.5 g) was used in the next step without further purification.

The 3-oleoyl-*sn*-glycerol, a soft paste, was found to be soluble at room temperature in chloroform, diethyl ether, benzene and petroleum ether, and insoluble in water.

1-O-Triphenylmethyl-3-Oleoyl-*sn*-Glycerol (VIII), 1-O-Triphenylmethyl-2-Linolenoyl-3-Oleoyl-*sn*-Glycerol (IX) and 2-Linolenoyl-3-Oleoyl-*sn*-Glycerol (X)

These were prepared by the tritylation, acylation and cleavage procedures employed for compounds I, II and III.

Preparation of 1-O-Triphenylmethyl-3-Stearoyl-*sn*-Glycerol (XI)

The 2-linoleoyl-3-stearoyl-*sn*-glycerol was obtained by the same sequence of reactions as 2-linolenoyl-3-oleoyl-*sn*-glycerol from D-mannitol via 3-stearoyl-*sn*-glycerol (14). (See reaction scheme, Fig. 2).

1-O-Triphenylmethyl-2-Linoleoyl-3-Stearoyl-*sn*-Glycerol (XII) and 2-Linoleoyl-3-Stearoyl-*sn*-Glycerol (XIII)

These were prepared by the acylation and cleavage procedures employed for compounds II and III.

The removal of the triphenylmethyl protecting groups with silicic acid and boric acid mixture proceeded without acyl migration. All the detritylated products were examined on the TLC and no 1,3-diacyl-*sn*-glycerol could be detected on the chromatographic plates.

These diacyl-*sn*-glycerols containing polyunsaturated chains are sensitive to oxidation. Therefore they must be kept under a nitrogen atmosphere. To prevent the loss of optical activity they were stored under anhydrous conditions at -15 C.

All the analytical values, yields and the physical properties of the intermediates and of the 1,2-diacyl-*sn*-glycerols and 2,3-diacyl-*sn*-glycerols described in this paper are summarized in Tables I and II.

DISCUSSION

The chemical synthesis of optically active 1,2-diacyl-*sn*-glycerols and 2,3-diacyl-*sn*-glycerols containing two dissimilar fatty acid substituents, either a saturated in 1 position and a polyunsaturated in 2 position or monounsaturated in 3 position from D-mannitol and L-mannitol via 1-acyl-*sn*-glycerol and 3-acyl-*sn*-glycerol, respectively, follows a relatively simple procedure. However since L-mannitol is not commercially available it was deemed desirable to devise methods which permit the synthesis of mixed acid, saturated and polyunsaturated, 1,2-diacyl-*sn*-glycerols and 2,3-diacyl-*sn*-glycerols from commercially available D-mannitol.

The procedure developed by Pfeiffer et al. (13) cannot be adapted to the synthesis of mixed acid diacyl-*sn*-glycerols because the synthesis of mixed acid diacyl-*sn*-glycerols requires a step by step introduction of the two dissimilar fatty acid substituents (see reaction scheme, Fig. 1 and 2).

With these restrictions in mind, procedures have been developed that permit the synthesis of both enantiomeric forms of mixed acid, saturated and polyunsaturated, 1,2-diacyl-*sn*-glycerols and 2,3-diacyl-*sn*-glycerols, respectively, from D-mannitol.

Recent studies of the positional distribution of fatty acids in 1,2-diacyl-*sn*-glycerols of 3-phosphatidylcholine and 3-phosphatidylethanolamine by Lands and Hart (22), Brandt and Lands (23), and by Kuksis et al. (24-27) have indicated that the saturated fatty acids are in the 1 position, while all the polyunsaturated fatty acids are esterified in the 2 position of the glycerol moiety. This is also true for the nonrandom stereospecific distribution of fatty acid residues in the triacyl-*sn*-glycerols (28-31).

As most of the natural 1,2-diacyl-*sn*-glycerol structures contain saturated and a polyunsaturated fatty acid substituents, it seemed of interest to continue this work by synthesizing the following representatives: 1-Palmitoyl-2-oleoyl-, 1-palmitoyl-2-linoleoyl-, 1-palmitoyl-2-linoelnoyl- and 1-palmitoyl-2-arachidonoyl-*sn*-glycerols.

The synthesis of these 1,2-diacyl-*sn*-glycerols is now in progress in this laboratory.

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Effects of Pure Oxygen Atmosphere in Vivo on Plasma Lecithin-Cholesterol Acyltransferase Reaction

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ABSTRACT

Exposure to pure oxygen atmospheres for more than three days resulted in loss of red blood cell mass in astronauts in some flights. Oxidation of lipid or protein membrane components may account for this phenomenon, since tocopherol deficient red blood cells have been shown to hemolyze on exposure to elevated oxygen pressures in vitro and in vivo. An additional contributing cause to the induced red cell loss may be the inhibition of the plasma lecithin-cholesterol acyltransferase reaction by oxygen, since congenital absence of the enzyme is associated with anemia. This possibility was explored in eight human subjects who lived in a sealed environmental chamber and breathed 100% oxygen at 258 mm Hg pressure for 30 days. In their plasma a 40% decrease in the conversion of free cholesterol to ester was demonstrated in vitro after four weeks' exposure to 100% oxygen. Plasma cholesteryl ester concentration was also decreased. Red blood cell mass was reduced and erythropoiesis was depressed. These findings support the hypothesis that the anemia induced by exposure to 100% oxygen was caused in part by decreased synthesis of cholesteryl esters secondary to an inhibition of the plasma lecithin-cholesterol acyltransferase reaction.

TABLE I

Composition of Daily Diet Fed Subjects During Study[a]

Components of Diet	Grams	Calories
Carbohydrate	428.6	1,714
Protein	132.2	529
Fat	84.3	759
		3,002
Polyunsaturated fatty acids		
Linoleate	6.0	
Linolenate	0.6	
Arachidonate	0.6	
Antioxidants		
Vitamin E	6.7 i.u.[b]	
BHT, BHA[c]	trace[d]	

[a]Composition is based on the intake of a 70 kg male fed 43 cal/kg/day during study. The values are calculated according to the composition of food reported by Hardinge, M., and H. Crooks, J. Amer. Diet. Ass. 34:1065-1071 (1958).

[b]Value was determined in triplicate on homogenized meal by the method of Finn Bro-Rasmussen and W. Hjarde, Acta Chem. Scand. 11:34-43 (1957), by Wisconsin Alumni Research Foundation.

[c]Abbreviations: Butylated Hydroxy Toluene, Butylated Hydroxy Anisole.

[d]No added antioxidants were given, only those amounts present as preservative in foods. Quantity not determined chemically.

INTRODUCTION

One of the biological problems associated with space flights has been the loss of red blood cell mass in American astronauts in certain flights after breathing 100% oxygen at 258 mm Hg pressure for more than three days in flight (1). Oxidation or peroxidation of constituents of the erythrocyte membranes may contribute to the phenomenon, since exposure of tocopherol deficient red blood cells to a pure oxygen atmosphere at high pressures either in vitro or in vivo results in appreciable hemolysis (2,3). Oxidative damage to enzyme systems in erythrocyte membranes must be considered. Ferber et al. showed a 50% decrease in acyl CoA lysolecithin-0-acyltransferase activity during aging of erythrocytes (4).

Another possible cause of loss of red blood cell mass in astronauts may be associated with oxygen-induced inhibition of the plasma lecithin-cholesterol acyltransferase reaction of Glomset (5-7). Norum and Gjone have reported a normocytic normochromic anemia associated with the congenital absence of the plasma lecithin-cholesterol acyltransferase activity (8-10). Mickel and Foulds have shown this enzymatic activity to be markedly inhibited in the presence of 0.1 M hydrogen peroxide (11). The congenital absence of this enzyme and anemia may be coincidental. However, should this enzymatic activity be required for normal hemopoiesis, an oxygen-induced reduction in activity might result in anemia. This possibility was explored in eight human subjects who lived

in a sealed environmental chamber and breathed 100% oxygen at 258 mm Hg pressure for 30 days.

MATERIALS AND METHODS

On five different occasions, 1 ml plasma samples were obtained from each of eight hematologically normal young military men (ages 18-24) who, as informed volunteers, served as subjects. Blood samples were taken at approximately the same time of day following an overnight fast, and collected in citric acid-dextrose to prevent clotting. Values were corrected for dilution of the plasma based on the individual hematocrit. Heparin was not used as anticoagulant since it has been shown to inhibit the plasma lecithin-cholesterol acyltransferase reaction in vivo (12) and to result in alteration of phospholipids to the lyso-form in vitro (13).

All of the test subjects consumed a diet consisting of reconstituted freeze-dried materials. Water was allowed ad libitum.

Control samples 1 and 2 were obtained after one and two months, respectively, on the controlled diet. During this time all subjects participated in the same exercise program. The third and fourth samples were taken after the subjects were placed in a sealed environmental chamber constructed for human use, and the atmosphere converted to 100% oxygen at 258 mm Hg pressure. The subjects were exposed to 100% oxygen in the chamber for 30 days. The fifth sample was taken during the second week after return to an air atmosphere at ground level atmospheric pressure, while still on the controlled diet.

During the oxygen exposure period four of the subjects were engaged in active exercise, while the other four were inactive at bed rest. The active group exercised for two half hour periods each day on a bicycle ergometer while heart rate was monitored. Exercise effort was built up to a peak heart rate of 150-160 beats per minute and maintained for 5-6 min during each exercise period. There was no separation into active and inactive groups prior to entering the chamber.

Daily food consumption of active and inactive subjects was 43 and 46 calories per kilogram lean body mass respectively (Table I).

To assay plasma lecithin-cholesterol acyltransferase activity, 1 ml of plasma from each individual was incubated at 37 C for a period of 24 hr with a 4-^{14}C-cholesterol (New England Nuclear Corporation. Specific activity: 57 mc/mM. Radiochemical purity: greater than 97%). 4-^{14}C-cholesterol was added to the incubation vial in benzene in the amount of 0.05 μc (8.8 x 10^{-4} μM or 0.32 μg) per ml plasma, and

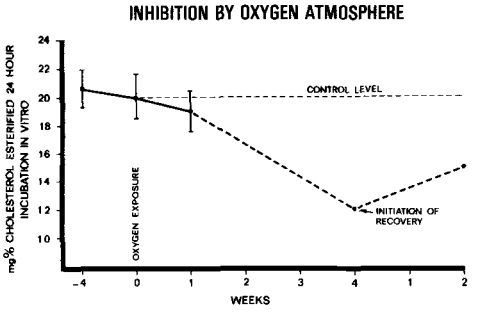

FIG. 1. The results are plotted as the mean mg/100 ml free cholesterol esterified during 24 hr incubation of plasma in vitro at 37 C ± standard error of the mean. Active and inactive groups were not significantly different and are represented together. The first two samples represent control values obtained a month apart while on the controlled diet described in Table I. The third sampling was during the first week of exposure to pure oxygen at 5 psi or 258 mm Hg, while the fourth sample was obtained during the fourth and final week of exposure. The fifth or recovery sample was obtained during the second week at ground level pressure breathing atmospheric air. The mg/100 ml cholesterol esterified during the fourth week of exposure is significantly less than all other values except the recovery measurement, which in turn is significantly less than the control values. Refer to text.

the substrate evaporated to dryness by a stream of nitrogen, prior to adding the plasma and capping the vial. The quantity of labelled cholesterol is negligible chemically in view of the much greater amount of endogenous free cholesterol (250 to 500 μg/ml) in the plasma.

After incubation for 24 hr at 37 C, with shaking, 0.5 ml of incubated plasma was extracted with 24.5 ml chloroform-methanol (2:1 v/v) in a 25 ml volumetric flask. Extraction was allowed to occur overnight at room temperature in the dark. An aliquot of 20 ml was evaporated to dryness under reduced pressure and temperature, and the residual lipids redissolved in purified *n*-hexane.

The extracted lipids were added to 0.1 g silica gel columns in 1 ml *n*-hexane. Separation of the total cholesterol into free and ester fractions was accomplished by silica gel column chromatography, as adapted by Leeder and Clark (14) from the method of Hirsch and Ahrens (15). The fractions were evaporated to dryness and counted in a Packard Tri-Carb Scintillation Counter, using 8 ml toluene (Mallinckrodt Chemical Company, scintillation grade), containing 5 g/liter PPO (2,5-Diphenyloxazole, Packard Instrument Company) and 0.1 g/liter dimethyl POPOP (1,4-bis-2[4-methyl-r-phenyloxazolyl]-benzene, Packard Instrument Company).

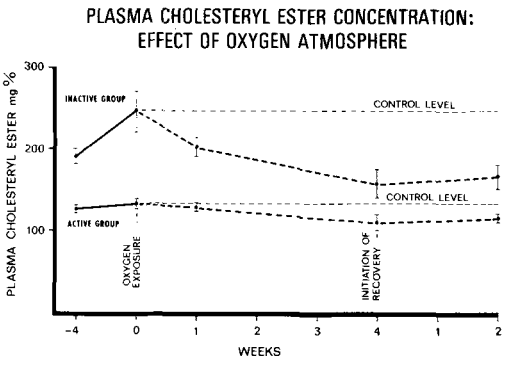

FIG. 2. The results are plotted as the mean plasma cholesteryl ester concentration for the active and inactive groups, ± standard error of the mean. The upper line represents values for the group of four subjects who were inactive during oxygen exposure; the lower line represents values for the group of four subjects who were active during oxygen exposure. The increase in plasma cholesteryl ester concentration during the second control sampling, compared to the first, is possibly a dietary effect since the first sample was obtained one to two weeks after instituting the controlled diet. The plasma cholesterol values of the two groups are significantly different prior to separation into active and inactive groups during oxygen exposure. However the groups are significantly different in initial body weight (Table II), such that differences noted between the two groups may be unrelated to activity.

Plasma lecithin-cholesterol acyltransferase activity was measured by the percentage of free cholesterol converted to cholesteryl ester, where the radioactivity in the cholesteryl ester fraction was expressed as the percentage of the total recovered radioactivity. Only negligible radioactivity was coated to the side of the incubation vial following incubation. However variability in the distribution of exogenous cholesterol to the plasma lipoproteins in the various samples was not examined. It is assumed that there is little variability.

The concentration of free and ester cholesterol present in the plasma prior to incubation was determined by the colorimetric method of Rosenthal et al. (16).

RESULTS

Exposure to 100% oxygen atmosphere at 258 mm Hg pressure resulted in a decrease in the activity of the plasma lecithin-cholesterol acyltransferase as measured in vitro by radiochemical assay (Fig. 1). There was no statistically significant difference in assay results from the active and inactive subjects. The inhibition by oxygen was not induced rapidly; the decrease in activity during the first week of exposure was not statistically significant. The

TABLE II

Variation in Initial Weight (Kg) of Subjects[a]

Active	Inactive
76.0	69.0
80.5	64.5
65.5	63.6
74.0	61.0
74.0 ± 3.1	64.5 ± 1.7

[a]The active and inactive groups are significantly different ($P < 0.05$) in initial weight.

level of activity by the fourth week of exposure in the chamber was significantly lower ($P < 0.001$) than any of the preceding values. Although the mean activity during the second week of recovery is a larger number than the fourth week of oxygen exposure, the two levels are not significantly different. However the value during recovery was significantly lower than the initial control values ($P < 0.001$).

Plasma cholesteryl ester levels reflect the changes determined by the in vitro assay (Fig. 2). Statistical analyses for the plasma cholesteryl ester concentrations suggest that the active and inactive groups differ not only in the level of the cholesteryl ester ($P < 0.01$) during the control period when there was presumably no difference in their activity, but also in variability. Since the groups are statistically different in initial body weight (Table II), the differences noted between the two groups may be unrelated to activity. Despite the differences between the two groups, both show a similar decreasing trend in plasma cholesteryl ester concentrations during exposure to oxygen.

A significant decrease in plasma cholesteryl ester concentration is seen by the fourth week in both groups. For the active group the value of the fourth week sampling was significantly less ($P < 0.0025$) than either control sampling. The fourth week sampling for the inactive group was significantly less ($P < 0.0005$) than the second control sampling, and less ($P < 0.025$) than the first week of exposure, but not significantly less than the first control nor the recovery measurement. For these inactive subjects the increase in level of the second control sampling over the first is significant ($P < 0.0001$). This observation is unexplained. The increase may reflect an effect of diet on the plasma cholesteryl ester level, more prominent in less muscular individual, i.e., inactive group (Table II).

DISCUSSION

In this study a significant decrease in the activity of the plasma lecithin-cholesterol acyl-

transferase was found after exposure in vivo to an oxygen atmosphere at 258 mm Hg pressure. The decrease in atmospheric pressure is not likely to be involved in the observed effect; it is considered that the decrease in enzymatic activity is the result of exposure to a pure oxygen atmosphere.

The inhibition of enzymatic activity by oxygen may be comparable to the effect of hydrogen peroxide on the enzyme. We have shown that 0.1 M hydrogen peroxide inhibits markedly the serum lecithin-cholesterol acyltransferase activity in vitro (11). Similarly hydrogen peroxide and peroxidized lipids result in inhibition of the plasma lecithin-cholesterol acyltransferase reaction by affecting sulfhydryl groups (H.S. Mickel, E.L. Foulds and D.A. Clark, in preparation). Similar concentrations of hydrogen peroxide were shown by Clark et al. to result in alterations in serum lipoproteins (17). Since the acyltransferase activity is inhibited by sulfhydryl blocking agents and is reversibly inhibited by *p*-hydroxymercuribenzoate (5), the in vivo effect of oxygen may parallel that of hydrogen peroxide and peroxidized lipids in altering sulfhydryl groups necessary for enzymatic activity.

Oxidation of these sulfhydryl groups may be the result of direct attack by molecular oxygen. Alternately a decrease in the acyltransferase activity may result from the peroxidation of polyunsaturated fatty acids in the plasma lipids. Destruction of sulfhydryl groups occurs in the presence of peroxidized lipids (18,19), and acyltransferase activity has been shown to be abolished in the presence of peroxidized lecithin.

There may be an association between reduced plasma lecithin-cholesterol acyltransferase activity and anemia. The congenital absence of the plasma enzyme is associated with very low levels of circulating cholesteryl ester, markedly elevated free cholesterol concentration, absence of α-lipoproteins, lipid deposits in the cornea, normocytic-normochromic anemia with shortened erythrocyte survival time, and proteinuria.

In this study a reduction in the plasma lecithin-cholesterol acyltransferase activity was associated with a reduction in plasma cholesteryl ester concentrations, a minimal reduction in high density lipoproteins, and anemia, but no observed proteinuria nor corneal lipid deposits.

The red blood cell mass decreased approximately 13% and the hematocrit dropped approximately 5% during exposure of the group to oxygen. Since mean red blood cell indices calculated from hematocrit, hemoglobin concentration, and red blood cell count were unchanged, the anemia was normochromic-normocytic. Unchanged or suppressed reticulocytosis indicated suppressed erythropoiesis during exposure to oxygen. At least a fourfold increase in reticulocytosis occurred during recovery. Reduction in erythropoiesis was also indicated by a steady decline in hemoglobin with no significant change in endogenous carbon monoxide production. Shortened erythrocyte survival curves during exposure to oxygen suggest that a hemolytic process was present. Hence there was evidence for both decreased production and some increased destruction of erythrocytes on exposure to oxygen. It is attractive to postulate that the primary product of the acyltransferase reaction, cholesteryl linoleate, is required for erythropoiesis. This study showed a decrease in the rate of formation of cholesteryl ester by in vitro radiochemical assay, with an associated decrease in plasma cholesteryl ester concentration in individuals exposed to the 100% oxygen atmosphere. Therefore a decrease in the total amount of cholesteryl linoleate formed under these conditions seems probable. The decrease could be accentuated by additional peroxidative attack on cholesteryl linoleate during exposure to an oxygen atmosphere. The net effect would be to decrease the amount of cholesteryl linoleate available to tissues.

Decreased availability of cholesteryl linoleate might decrease function of hematopoietic or other tissues by depriving them of the polyunsaturated fatty acid. In other words polyunsaturated fatty acid metabolism may be integrally related to cholesteryl ester metabolism. Polyunsaturated fatty acid deficiency in animals has been shown to result in increased red blood cell fragility (20). There is a marked hyperemia of the kidneys resulting in proteinuria and gross hematuria in some animals (20). Polyunsaturated fatty acid deficiency appears similar in some respects to the syndrome associated with the congenital absence of the plasma lecithin-cholesterol actyltransferase activity.

Polyunsaturated fatty acids may be required for optimal membrane synthesis, possibly as a membrane constituent. The incorporation of cholesteryl esters into the early myelin sheath during development in the human (21) suggests that fatty acids may be introduced into developing cellular membranes, in part, as the cholesteryl esters.

A decrease in available polyunsaturated fatty acids could also result in a decrease in prostaglandin biosynthesis. Linoleic acid had been shown to be a precursor of eicosatrienoic acid (22) and eicosatetraenoic acid (23), which in

turn are precursors of prostaglandins (24). If cholesteryl ester is required for optimal availability of polyunsaturated fatty acids for prostaglandin biosynthesis, a generalized decrease in prostaglandin formation would result from inhibition of cholesteryl linoleate biosynthesis by oxygen atmospheres. Such a decrease in prostaglandin biosynthesis would be expected to result in diverse endocrine and metabolic changes affecting many organ systems.

Despite this speculation it is possible that prolonged exposure to pure oxygen atmospheres may cause diverse metabolic effects by direct peroxidation of double bonds in labile lipids or by oxidation of sulfhydryl groups or other reactive sites on enzymes, or both.

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Stereospecific Hydration of Unsaturated Fatty Acids by Bacteria[1]

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ABSTRACT

Three new 10-hydroxy fatty acids, all optically active, have been prepared by the anaerobic microbiological hydration of a *cis*-9 double bond. Substrates that formed these new hydroxy fatty acids are linoleic, linolenic, and ricinoleic acids. The hydroxyl group has the D configuration and the methyl esters are levorotatory. Infrared, mass spectral, specific rotation and ultraviolet data on these compounds were determined. There was no migration of the unreacted double bonds at C_{12} and C_{15} in linoleic or linolenic acids. The presence of a double bond in the 10-hydroxy fatty acids significantly increased the optical rotation of the methyl esters. The hydratase enzyme showed unusual specificity among Δ^9 unsaturated acids. While it hydrates methylene interrupted and hydroxy unsaturated acids, it failed to hydrate either 9-decenoic, 12,13-epoxy- or 12-keto-*cis*-9-octadecenoic acids or sterculic acid.

INTRODUCTION

In 1962, Wallen et al. (1) found that the unidentified *Pseudomonas* sp. NRRL B-2994 (ARS Culture Collection, Northern Regional Laboratory) converted oleic acid to 10-hydroxystearic acid. Davis et al. (2) studied the production and activity of the highly specific hydratase induced in this isolate and in another, *Pseudomonas* sp. NRRL B-3266. These organisms anaerobically hydrated a number of *cis*-9 fatty acids. The products from palmitoleic and oleic acids were optically active (-)-10 D-hydroxy acids. Davis reported that Δ^9 fatty acids not having the *cis* configuration or lacking a free (unesterified) carboxyl group were not hydrated.

Dienoic acids with a *cis*-9 bond should yield unsaturated hydroxy acids isomeric with ricinoleic acid. Gunstone (3) reported the presence of 9-hydroxy-12-octadecenoic acid in *Strophanthus sarmentosus* seed oil, but there are no reports in the literature of an unsaturated 10-hydroxy fatty acid. Hydration of a *cis*-9 unsaturated hydroxy fatty acid, such as ricinoleic acid, by the enzyme of these organisms would be expected to yield a dihydroxy product. We report here the characterization of several new optically active hydroxy fatty acids produced by the anaerobic activity of resting cells of *Pseudomonas* sp. NRRL B-3266. The specificity of the hydratase is also further delineated.

METHODS

Fatty acids of high purity were purchased from The Hormel Institute. The 9-decenoic acid was prepared by 24 hr oxidation of 9-decenol with chromic acid in glacial acetic acid at room temperature. The presence of the unsaturated bond was confirmed by bromine addition and by infrared (IR) analysis. Saponification (4) of sterculia oil extracted from *Sterculia foetida* nuts gave crude sterculic acid. The 12-keto-oleic acid was prepared by the oxidation of ricinoleic acid with potassium dichromate in sulfuric acid according to the method of Nichols and Schipper (5). All methyl esters were prepared with diazomethane.

The yeast extract salts fermentation medium and procedures used are those described by Davis et al. (2). Bacteria were grown in this medium with inducer levels of substrate acid for 24 hr under aerobic conditions; the substrate was then added and the flask atmosphere was made anaerobic by replacement of air with prepurified nitrogen (less than 8 ppm O_2). After the desired incubation period small fermentations (50 ml) were acidified and extracted with ether in a liquid liquid continuous extractor for 6 hr; large fermentations (5-10 liters) were acidified, slurried with Hy-flo Super Cel, and filtered by suction through a filter bed of the same material to give a clear amber aqueous filtrate. The filter bed was then suspended in ether and washed several times by decantation to recover the entrapped fatty acids. A small sample of the recovered acids was then examined by gas chromatography of their methyl esters. Solid products were recovered and purified by fractional crystallization from cold hexane at -15 C.

Chromatography

Liquid products were separated as their

[1]Presented at the AOCS Meeting, San Francisco, April 1969.

[2]No. Marketing and Nutrition Res. Div., ARS, USDA.

TABLE I

Specific Rotation of Methyl Esters of Hydroxy Fatty Acids at 550 nm[a]

Ester	$[\alpha]_{550}^{26}$
Methyl 10-hydroxystearate	-0.03
Methyl 10,12-dihydroxystearate	-0.41
Methyl 10-hydroxy-*cis*-12, *cis*-15-octadecadienoate	-5.7
Methyl 10-hydroxy-*cis*-12-octadecenoate	-6.4
Methyl ricinoleate	+6.8
Dimethyl 3-hydroxy-dodecanedioate[b]	+0.40

[a]In methanol at 26 C.

[b]Negative rotation at 340 nm.

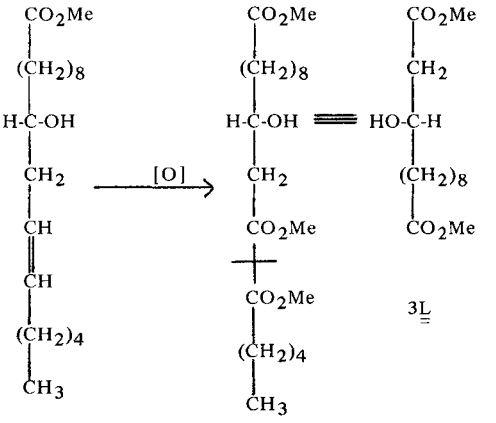


FIG. 1. Configuration of the dicarboxylic hydroxy acid from von Rudloff oxidation of methyl 10-hydroxy-*cis*-12-octadecenoate.

methyl esters by silicic acid column chromatography. All silicic acid columns contained a 40 x 480 mm bed of Bio-Sil A (100-200 mesh); esters were eluted with 10% ether in hexane.

Qualitative analysis of fermentation extracts was done by gas chromatography of methyl esters at 250 C for 1 min followed by temperature programming at 15 C/min to 300 C on a Model 700 F&M instrument equipped with dual 4 ft columns of 20% OV-17 on 80-100 mesh Chromosorb W pretreated with hexamethyldisilazane. A Disc integrator was used to approximate product yields.

Thin layer chromatograms were developed with 10% ether in hexane on 20 x 20 cm Eastman Chromagram sheets. Spots were visualized under ultraviolet (UV) light after first spraying with a 0.2% methanol solution of 2',7'-dichlorofluorescein.

Spectrometry

Mass spectra of the methyl esters of products were obtained on a Model 12-90 G Nuclide mass spectrometer. The spectra were interpreted according to the fragmentation scheme for hydroxy fatty acids (6). IR analyses were run by internal reflection spectroscopy on a Beckman IR-8 instrument. Films of samples were formed on the surface of a KRS-5 plate (Wilks Scientific Corp., South Norwalk, Conn.) by evaporation from applied hexane or acetone solutions.

Ultraviolet Spectra

The UV spectra were measured at room temperature in a Cary 14 recording spectrophotometer from 350 to approximately 200 nm. The cell path varied from 0.2-2.5 cm; the concentration was usually 5%. The solvent blank was subtracted from the observed optical density of the solution.

Optical Rotation

A Cary 60 recording spectropolarimeter was used to measure the specific rotation of each sample at 550 nm in a 10 cm cell at 26 C. The concentration of the solution was usually 5%. The instrument was calibrated with a sucrose solution (National Bureau of Standards) and a quartz control plate as described by Wu and Cluskey (7). A constant band pass of 1.5 nm was selected for the experiments. The solvent blank was subtracted from the observed rotation of the solution.

RESULTS AND DISCUSSION

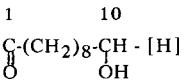
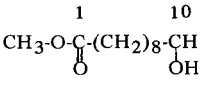
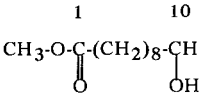
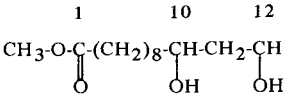
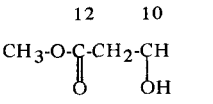
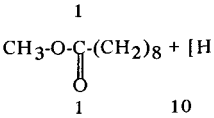
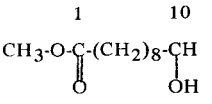
When linoleic and linolenic acids serve as substrates an inert atmosphere is necessary to decrease the probability of autoxidation. Under these conditions subsequent separation and examination of products and of starting material showed that no significant oxidation had occurred.

Linoleic Acid

Linoleic acid was converted to 10-hydroxy-*cis*-12-octadecenoic acid in 20 mole per cent yield. The product was separated as its methyl ester on a silica gel column and gave a single peak on a gas chromatogram. The remainder of the material recovered was unchanged substrate. The 10-hydroxy acid and its methyl ester are oils at room temperature. The methyl ester had a mp of -7 to -5 C. Methyl 10-hydroxy-*cis*-12-octadecenoate is isomeric with methyl ricinoleate and showed an IR spectrum nearly identical to it. Prominent features included a strong hydroxyl band at 3450 cm^{-1}, bands at 3000 cm^{-1} and 1650 cm^{-1} for *cis* unsaturation, but none at 967 cm^{-1} (*trans*), and

TABLE II

Mass Spectral Data of Hydroxy Esters

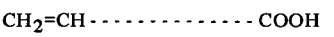
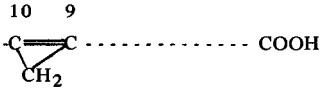
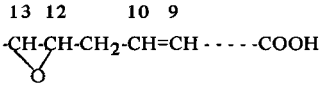
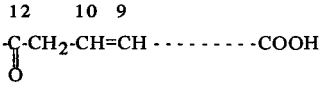
Compound	Fragments	Mass
Methyl 10-hydroxy-*cis*-12,*cis*-15-octadecadienoate mol wt 310		169
Methyl 10-hydroxy-*cis*-12-octadecenoate mol wt 312		201
Methyl 10,12-dihydroxystearate mol wt 330		201
		245
Dimethyl 3-hydroxydodecanedioate mol wt 274		103
		172
		201

at 1737 cm^{-1} for an ester carbonyl. The methyl ester is levorotatory as is the methyl 10-hydroxystearate obtained from the hydration and esterification of oleic acid. This unlike methyl ricinoleate which is dextrorotatory but gives a levorotatory reduction product (8). Schroepfer and Block (9) determined that the hydroxyl group of levorotatory methyl 10-hydroxystearate produced by the microbial hydration of oleic acid had the D configuration. Tulloch and Spencer (10) showed that the hydroxyl group of levorotatory monohydroxy fatty acids also has the D configuration. By reduction of 10-hydroxy-*cis*-12-octadecenoic acid (formed by the hydration of linoleic acid), we obtained 10-hydroxystearic acid whose methyl ester was levorotatory, thereby proving that the hydroxyl group of the 10-hydroxy unsaturated acid has the D configuration. The rotation values are given in Table I. Values for methyl ricinoleate are included for comparison. A mass spectrum indicated fragmentation as shown in Table II and confirmed the presence of the hydroxyl group at carbon 10.

Acidic products from the degradation of methyl 10-hydroxy-*cis*-12-octadecenoate by the von Rudloff procedure (11) were esterified and identified as dimethyl 3-hydroxydodecanedioate and methyl hexanoate. The 3-hydroxy C_{12} dicarboxylic ester was identified by IR spectroscopy, comparative gas chromatographic retention times, and mass spectroscopy. Since the location of the hydroxyl group of the dicarboxylic ester was established at C-3, the location of the double bond of the original C_{18} unsaturated acid was confirmed at the C-12 position. The odorous hexanoic acid was identified by comparative gas chromatography of its methyl ester against methyl hexanoate. The 3-hydroxy dicarboxylic acid degradation product was dextrorotatory. Precedents in the literature (10) indicate that on this basis the 3-hydroxy dodecanedioic acid has the L configuration for the hydroxyl group. The reaction sequence in Figure 1 shows the relationships of the 3-hydroxyl group to the nearest carboxyl group as a result of the oxidative cleavage reaction.

TABLE III

Inactive Unsaturated Fatty Acid Substrates

Acid	Structure
9-Decenoic (C_{10})	
Sterculic (C_{18})	
Vernolic (C_{18})	
12-Keto-oleic (C_{18})	

Linolenic Acid

Autoxidation was insignificant in fermentations of linolenic acid under anaerobic conditions. Bacterium B-3266 gave 10-hydroxy-*cis*-12,*cis*-15-octadecadienoic acid in 21 mole per cent yield from linolenic acid. After esterification of the crude products, methyl 10-hydroxy-*cis*-12,*cis*-15-octadecadienoate was separated on a silica gel column and gave a single gas chromatographic peak with the expected retention time of 8.8 min. Although the product ester gave IR bands very similar to those of the linoleic acid-derived product, a stronger band at 3050 cm^{-1} but none at 967 cm^{-1} (*trans*) indicated more *cis*-unsaturation than in the product from linoleic acid. Mass spectroscopy confirmed the location of the single hydroxyl group on carbon 10 of the chain (Table II). The purified C_{18} methyl ester is an oil, mp -15 to -14 C. Its rotation is given in Table I. Although Christie and Holman (12) report that *cis*-12,*cis*-15-octadecadienoic acid is a solid which melts at 18 C, the 10-hydroxy derivative of this acid was an oil.

Esterification of the products from the von Rudloff degradation of methyl 10-hydroxy-*cis*-12,*cis*-15-octadecadienoate gave dimethyl 3-hydroxydodecanedioate and a gas chromatographic peak with the same retention time as dimethyl malonate. Data obtained by IR, gas chromatography, and mass spectroscopy of these derivatives confirmed the location of double bonds at the C_{12} and C_{15} positions of the parent product.

Ricinoleic Acid

The anaerobic fermentation of ricinoleic acid by B-3266 gave a 41 mole per cent yield of optically active 10,12-dihydroxystearic acid. IR analysis showed a strong hydroxyl absorption at 3300 cm^{-1} but no carbonyl band except that for the carbomethoxy group at 1735 cm^{-1}. Bands for *cis*- or *trans*-unsaturation were absent. Although 9,10-dihydroxystearic acid melts at 99 C, our free acid melted at 66.5-67.0 C. This behavior is attributed to the 1,3-diol positions of the two hydroxyl groups relative to each other. The methyl ester had a low optical rotation (Table I) and gave a single spot of low R_f value (0.25) on a thin layer chromatogram developed with 30% ether in hexane. It melted at 53-54 C. A mass spectrum of methyl 10,12-dihydroxystearate gave representative fragments consistent with the expected fragmentation pattern for a 10,12-dihydroxy fatty acid (Table II). The hydroxyl group of ricinoleic acid has the D configuration (13) and the 10 (-) hydroxyl group introduced into the molecule by microbiological processes has the D configuration. We therefore surmise that the hydrated product from ricinoleic acid is (-)-10 D, 12 D-dihydroxystearic acid.

The methyl ester of 10,12-dihydroxystearic acid was labile at high temperatures. When a mass spectrum was prepared at an all glass molecular leak inlet at 200 C, no molecular weight fragments greater than mass 43 were produced; whereas a probe inlet temperature of 125 C gave a satisfactory spectrum. When sam-

ples of ester were heated in a tube to their boiling point, IR spectra were similar to those for methyl ricinoleate. Seemingly the ester loses a molecule of water between one of the hydroxyl groups and an adjacent hydrogen on a neighboring methylene group.

When ricinoleic acid was incubated with bacterium B-3266 under aerobic conditions, a small amount of product was formed whose retention time (following treatment with diazomethane) on a gas chromatogram was consistent for a monoketo or monohydroxy C_{12} ester. The overlapping IR bands at 1775 cm^{-1} and 1735 cm^{-1} (for lactone and ester, respectively), the hydroxyl band at 3410 cm^{-1}, and strong methylene absorption at 2850 cm^{-1} and 2910 cm^{-1} are supporting evidence for a mixture of methyl ester and a hydroxy lactone. The latter compound could result from oxidative degradation of the initial hydration reaction product, 10,12-dihydrixystearic acid, to form 4,6-dihydroxydodecanoic acid which then lactonizes. The band at 1735 cm^{-1} is probably a methyl ester, possibly of a dihydroxy or keto acid. An indication of some carbonyl absorption was seen at 1695 cm^{-1}. The presence of 4,6-dihydroxydodecanoic acid would be consistent with our earlier work (2) where 4-ketododecanoic acid was identified as a degradation product of 10-hydroxystearic acid in aerobic fermentations with B-3266. It is also consistent with the reported resistance (14) to bacterial degradation of C_{10} to C_{12} fatty acids containing a hydroxyl group on carbon 4, 5, or 6.

Miscellaneous Substrates

Anaerobic fermentations were tried with other fatty acids (Table III) having a Δ^9 bond. These acids were not converted into a product; no hydroxyl function could be detected with gas chromatography or by IR analysis.

Optical Rotation

The specific rotations of the methyl esters of hydroxy fatty acids were studied as a function of wavelength; values for 550 nm are shown in Table I. The specific rotation of methyl 10-hydroxystearate was considerably lower in magnitude compared with that of methyl 10,12-dihydroxystearate which in turn was considerably lower than those of other fatty acid esters studied.

The magnitude of specific rotation of the hydroxylated stearates is several times higher when two hydroxyl groups are present instead of one. This increase is to be expected because methyl 12 D-hydroxyoctadecanoate has a (-) rotation (8,10) as does methyl 10 D-hydroxyoctadecanoate. Thus each asymmetric center contributes to the total negative rotation.

The presence of a double bond in methyl 10-hydroxy-*cis*-12-octadecenoate increases the magnitude of specific rotation by 40-200 times over that of methyl 10-hydroxystearate where no double bond is present. However the location of a double bond and an increase in the number of double bonds from one to two have little influence on the magnitude of specific rotation, e.g., methyl 10-hydroxy-*cis*-12,*cis*-15-octadecadienoate, methyl 10-hydroxy-*cis*-12-octadecenoate, and methyl ricinoleate (methyl 12-hydroxy-*cis*-9-octadecenoate) all have comparable rotation values. With the exception of dimethyl 3-hydroxydodecanedioate, the magnitude of specific rotation increased with decreasing wavelength.

Ultraviolet Spectra

The hydroxy- and dihydroxystearates do not have absorption maxima or minima in the wavelength range (350-200 nm) studied. Methyl 10-hydroxystearate has low UV absorption at 273 nm, but methyl ricinoleate has two shallow minima and maxima close together in this region. Methyl 10-hydroxy-*cis*-12-octadecenoate and methyl 10-hydroxy-*cis*-12,*cis*-15-octadecadienoate have, respectively, only one maximum (274 and 272 nm) and one minimum (262 and 263 nm) each. The presence of an additional hydroxyl group, as in methyl 10,12-dihydroxystearate, increased UV absorption more than five times. The addition of a *cis* double bond increased UV absorption 9-64 times over that of methyl 10-hydroxystearate depending on the location of the bond. In methyl ricinoleate where the double bond is in the center of the molecule (*cis*-9) the increase is ninefold, but in methyl 10-hydroxy-*cis*-12-octadecenoate the absorption is increased 64 times. The presence of one or two double bonds in hydroxy fatty esters gave little difference in the UV absorption since values obtained for methyl 10-hydroxy-*cis*-12-octadecenoate and methyl 10-hydroxy-*cis*-12,*cis*-15-octadecadienoate were nearly identical.

DISCUSSION

The products from fermentations involving single and multiple double bond systems confirm the specificity of the hydro-lyase for hydration of the *cis*-9 position. A reaction product formed with 12-hydroxy-*cis*-9-octadecenoic (ricinoleic) but not with 12-keto-*cis*-9-octadecenoic (12-keto-oleic) acid or with 12,13-epoxy-*cis*-9-octadecenoic (vernolic) acid. The lack of an alkyl chain on the omega side of the double bond in 9-decenoic acid may have

prevented reaction, as did the steric effect of the cyclopropene ring system of sterculic acid. Also our C_{10} substrate may have been toxic to the microorganisms used here. On the basis of our negative results with these compounds, it appears that an alkyl chain on both sides of the Δ^9 bond is essential, and that these chains must be in a *cis* relationship to one another (2), rather than in a *trans* or a linear mode.

The bacterium B-3266 produces a hydrolyase specific for adding water to a *cis*-9 double bond. The direction of addition is specific with respect to the placement of the hydroxyl function at C_{10} in a D configuration and a hydrogen at C_9 in an L configuration (15). Other carbon-carbon double bonds do not block the reaction, nor does a hydroxyl at C_{12} inhibit it, although nearby epoxy and keto groups appear to do so. We believe that hydration is probably the first step in the microbiological degradation of *cis*-9 unsaturated fatty acids by this organism, followed by β-oxidation to a 4-keto- or -hydroxy-dodecanoic acid. The failure to detect products of shorter chain length supports the conclusions of Mizugaki et al. (14), who state that the degradation of hydroxy fatty acids by *E. coli* ceases when the carbon chain reaches a length of 10, 11, or 12, and the hydroxy group is on the sixth, fifth, or fourth carbon atom, respectively.

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Effects of Renal Factors on in Vitro Hepatic Cholesterol Synthesis in the Rat

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ABSTRACT

Two products derived from rat renal tissue have been shown to affect in vitro hepatic cholesterol synthesis. A pre-mevalonate inhibitor of hepatic cholesterol synthesis is associated with the membranes of the renal endoplasmic reticulum. It is stable at -75 C and at 4 C but is heat labile. A pre-mevalonate stimulator of in vitro hepatic cholesterol synthesis is located within renal lysosomes and can be prepared in a nonsedimentable form by extraction with hypotonic buffer. While it is stable at -75 C, it loses activity at 4 C. Both of these products appear to have a molecular weight in excess of 150,000 as determined by gel filtration.

INTRODUCTION

Effects of Renal Factors on Hepatic Cholesterol Synthesis

Abnormalities of lipid metabolism occur regularly in a wide spectrum of renal diseases. Even bilateral nephrectomy results in moderate hyperlipemia in many species (1). The most dramatic hyperlipemia occurs in the nephrotic syndrome, a symptom complex characterized by massive proteinuria, hypoproteinemia, hyperlipemia, edema and lipiduria. Although the mechanism of development of nephrotic hyperlipemia has not been definitively established, two major hypotheses have been proposed.

The most widely accepted hypothesis was proposed by Rosenman et al. (2) and subsequently extended by Marsh and Drabkin (3). In the presence of nephrotic hypoalbuminemia there is a generalized increase in the hepatic synthesis of proteins including lipoproteins. Low molecular weight proteins such as albumin are lost in the urine while high molecular weight proteins such as β-lipoproteins are not excreted in significant amounts. Accordingly Marsh and Drabkin explained the serum protein abnormalities of nephrosis on the basis of a "kinetic balance" between production in the liver and outflow in the urine, albumin being postulated as the key substance regulating protein synthesis by a negative feedback mechanism.

However Heymann et al. noted that nephrotic hyperlipemia can develop in the absence of significant hypoalbuminemia (4) and that unilateral nephrectomy partially corrects nephrotic hyperlipemia (5). They suggested that the kidneys influence hepatic cholesterol synthesis by the production during nephrosis of a renal "hyperlipemia inducing" substance, although they could not exclude a "lipemia depressing" substance which might be released from the kidneys in lesser quantities during nephrosis. Moreover Tracy (6) observed that renal protein excretion, renal tubular protein reabsorption, and hepatic lipid synthesis are directly related. He suggested that during periods of proteinuria the kidneys release a "lipemic factor" which stimulates the synthesis and release of hepatic lipoproteins.

While all of the above studies were performed using rats as experimental models, evidence of similar factors in another species was provided by Lagrue et al. (7), who studied serum lipids in the rabbit during unilateral and bilateral renal vein constriction. Despite comparable proteinuria and hypoproteinemia in both groups, hyperlipemia developed only after bilateral renal vein constriction. This simple experiment suggests that the development of hyperlipemia is related to an effect of nephrotic renal tissue rather than to hypoproteinemia. It also suggests that normal renal tissue can prevent nephrotic hyperlipemia, presumably by producing a substance which inhibits lipid synthesis.

The present report describes the identification and intracellular localization of renal lipemic and antilipemic factors which affect hepatic cholesterol synthesis in vitro.

METHODS AND MATERIALS

Aminonucleoside was obtained from Nutritional Biochemicals Co., Cleveland, Ohio. The phospholipids, bovine protein fractions, enzymes, coenzymes, and Bio-Gel resins used in this study were obtained from Calbiochem, Los Angeles, Calif. ^{14}C-labeled acetate and mevalonate were obtained as the sodium salts from New England Nuclear Corp., Boston, Mass. All

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TABLE I

The Effects of Rat Liver and Kidney Subcellular Fractions From Normal and Nephrotic Rats on the Incorporation of 2-^{14}C-Acetate Into Cholesterol by a Rat Liver Enzyme System

	Per cent of control activity ± 1 S.D. in the presence of subcellular fractions		
Liver subcellular fractions[a]	Saline injected	One week nephrotic	Two week nephrotic
Sucrose control	100 ± 18.2	100 ± 14.6	100 ± 13.3
Large granule fraction	3.1 ± 2.0	33.5 ± 20.6	4.0 ± 1.8
Small granule fraction	91.6 ± 15.2	121.1 ± 4.8	130.5 ± 8.1
Smooth Microsomes	70.3 ± 9.8	79.5 ± 2.7	124.5 ± 5.2
Cell sap	119.0 ± 10.5	104.5 ± 11.1	124.1 ± 13.8
Kidney subcellular fractions[a]			
Sucrose control	100 ± 29.6	100 ± 12.1	100 ± 4.6
Large granule fraction	1.2 ± 0.7	14.9 ± 3.5	2.1 ± 0.9
Small granule fraction	1.5 ± 1.2	3.9 ± 2.2	1.3 ± 0.5
Smooth microsomes	1.7 ± 0.7	0.5 ± 0.3	0.7 ± 0.2
Cell sap	144.2 ± 0.3	66.7 ± 14.9	117.8 ± 18.6

[a]All subcellular fractions were adjusted to 5 mg protein per incubation; four incubations per group; subcellular fractions were prepared from eight saline injected control rats and seven nephrotic rats at each time interval.

solvents and inorganic chemicals were obtained from Fisher Scientific Co., Pittsburgh, Pa.

Induction of Nephrosis

Nephrosis was produced in 250-300 g male albino rats by the intraperitoneal injection of 20 mg of Aminonucleoside in 4 ml of sterile saline (8). Controls were injected with sterile saline only. A nephrotic syndrome which regularly appeared within one week was confirmed by the findings of elevated pre-β and β-lipoprotein levels, hypercholesterolemia, hypoalbuminemia, and typical renal tubular changes as observed by light microscopy. Experimental animals sacrificed at one and two weeks after injection are termed, respectively, one week and two week nephrotic rats.

Subcellular Fractionations

Rat kidneys and livers were fractionated according to the following scheme. After Dounce homogenization (9) with 8 volumes of buffered 0.45 M sucrose (10), a cytoplasmic suspension was prepared by centrifugation at 650 *g* for 5 min. From this suspension the large granule fraction was sedimented at 10,000 *g* for 5 min. The resulting supernatant was then centrifuged at 15,000 *g* for 20 min to obtain the small granule fraction. A final centrifugation at 105,000 *g* for 60 min yielded smooth microsome as well as cell sap fractions.

In addition rat kidney lysosomes and mitochondria were prepared by the method of Shibko and Tappel (10) following Dounce homogenization. Rat liver lysosomes were prepared by the method of Sawant (11). Hypotonic extracts of kidney and liver fractions were prepared according to Contera (12). All fractions adjusted to known protein content were stored at -75 C prior to assay in the in vitro system.

In Vitro Assay System

The hepatic cholesterol synthesizing system used in this study was developed by Bucher (13), and modified by Knaus et al. (14) and Eskelson et al. (15). Rats were sacrificed by decapitation and their livers were perfused with 20 ml of ice cold pH 7.4, 0.1 M phosphate buffer containing 0.03 M nicotinic acid amide and 0.004 M magnesium chloride. The livers were then homogenized 1:1.5 (w/v) in the same buffer solution using a Potter homogenizer with trimmed pestle (15). Debris, whole cells and nuclei were discarded after centrifugation at 1000 *g* for 10 min. This supernate was then centrifuged at 9000 *g* for 30 min to obtain the supernatant enzyme source which was stored at -75 C until use. The incubation mixture contained 2.0 ml of this liver microsomal suspension, 0.5 ml of 1.0 M pH 7.0 phosphate buffer, 1.0 ml of test or control materials, 1.0 ml of 2-^{14}C acetate (2.5 μC in 5.0 μmoles) or 2-^{14}C mevalonate (0.4 μC in 1.0 μmoles) and 0.5 ml of coenzyme solution in a total volume of 5.0 ml. Each 0.5 ml of coenzyme solution contains 5.0 μmoles of ATP, 30.0 μmoles of $MgCl_2 \cdot 6H_2O$, 22.5 μmoles of glucose-1-phosphate, 30.0 μmoles of reduced glutathione, 0.2 μmoles of coenzyme A, 1.2 μmoles of NAD and 1.4 μmoles of NADP. The incubation mixture in a 50 ml glass-stoppered Erlenmeyer flask was

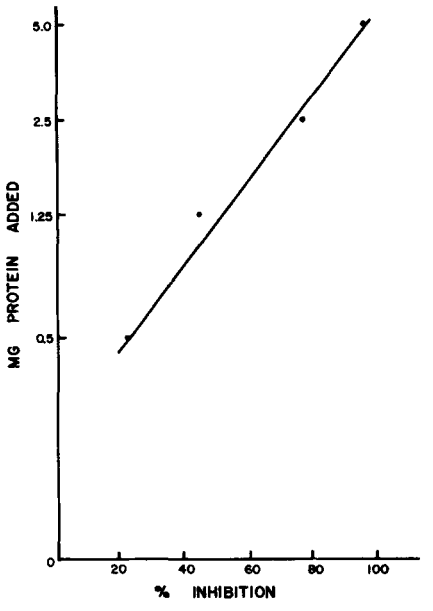

FIG. 1. The effects of various concentrations of rat kidney smooth microsomes on the incorporation of 2-^{14}C-acetate into cholesterol by a rat liver enzyme system.

oxygenated with a stream of 95% 0_2 for 10 seconds, stoppered, and incubated for 60 min in an oscillating Dubnoff shaker at 37 C.

After incubation, 1 ml of absolute ethanol containing 2 mg of cholesterol was added to each flask. The contents were poured into 25 x 200 mm test tubes and each flask was rinsed with 4 ml of 2.5 N methanolic KOH and the rinse added to the test tubes. Cholesterol esters were hydrolyzed for 75 min in a 75 C water bath. The tubes were allowed to cool and extracted three times with 10 ml of petroleum ether. To the combined petroleum ether extracts, approximately 2 g of anhydrous Na_2SO_4 was added. The tubes were shaken, the Na_2SO_4 allowed to settle, and the petroleum ether was decanted into a 50 ml centrifuge tube.

The petroleum ether was evaporated under a stream of N_2 in a 60 C water bath. The residue was taken up in 3 ml of acetone-ethanol-ether (4:4:1) solution. One drop of 1% phenolphthalein in ethanol, 0.1 ml of 2.5 N KOH in methanol, and 2 ml of a 1% tomatine solution (1 g tomatine, 55 ml H_20, 44 ml ethanol, and 1 ml glacial acetic acid) were added. The tube's contents were mixed by vortex and the sides of the tubes rinsed with a minimal amount of the acetone-ethanol-ether solution. Precipitation of the tomatinide was allowed to proceed overnight.

The tube's contents were then mixed by vortex, the sides of the tubes rinsed with acetone-ethanol-ether solution, and the precipitate collected by centrifugation at 1000 *g* for 5 min. The supernatant was discarded and the precipitate was washed twice by suspension in acetone-ethanol-ether mixture and subsequent centrifugation. The recovered tomatinide was dissolved in 2 ml of glacial acetic acid at 80 C. Five milliliters of scintillation fluid was added to each tube and the solution was transferred to counting vials. The tubes were then rinsed with 10 ml of scintillation fluid and this fluid was added to the appropriate vial. The determination of radioactivity of the cholesterol tomatinide was performed according to the method of Kabara et al. (16) as modified by Eskelson et al. (15).

Counts per minute were determined by liquid scintillation spectrometry. Efficiency was calculated by the channels ratio method. Disintegrations per minute (dpm), the mean dpm and standard deviation of a sample group and the significance of intergroup differences as measured by the Student t-test, were calculated with the aid of an IBM 1620 computer. Significance was accepted at $P < 0.05$.

TABLE II

Effect of Rat Kidney Microsomes and Purified Endoplasmic Reticulum Membranes on the In Vitro Incorporation of 2-^{14}C-Acetate Into Cholesterol by a Rat Liver Homogenate

Experimental conditions[a]	DPM ± 1 S.D.
Sucrose control	11,400 ± 1380
Rat kidney microsomes	2420 ± 570
Rat kidney e. r. membranes	120 ± 50

[a]Experimental groups contained 2 mg protein per incubation; four incubations per group.

TABLE III

Effects of Sedimentable and Nonsedimentable Fractions of Soniciated Rat Kidney Smooth Microsomes on the In Vitro Incorporation of of 2-^{14}C-Acetate Into Cholesterol by a Rat Liver Enzyme System

Experimental conditions[a]	DPM ± 1 S.D.
Sucrose control	3320 ± 26
Sonicated smooth microsomes, nonsedimentable[b]	390 ± 94
Sonicated smooth microsomes, sedimentable[b]	1380 ± 240

[a]Smooth microsomal fractions were adjusted to 2.5 mg protein per incubation; three incubations in control group, four incubations in each experimental group.

[b]Sedimentation was carried out at $10^5 g$ for 1 hr.

TABLE IV

Effects of Rat Kidney Microsomes on the Incorporation of 2-^{14}C-Acetate and 2-^{14}C-Mevalonate Into Cholesterol by a Rat Liver Enzyme System

Experimental conditions[a]	Substrate	DPM ± 1 S.D.
Sucrose control	Acetate	52200 ± 4100
Kidney microsomes[b]	Acetate	9460 ± 1680
Sucrose control	Mevalonate	208000 ± 5110
Kidney microsomes[b]	Mevalonate	180000 ± 5710

[a]Three incubations per group.

[b]Microsomes were derived from 1 g wet weight of renal tissue.

Chemical Determinations

Serum total cholesterol concentration was determined by the method of Abell et al. (17). Protein content of subcellular fractions was determined by the method of Lowry et al. (18). Total serum protein concentration was measured by the biuret procedure.

RESULTS

Effects of Kidney and Liver Subcellular Fractions

Table I depicts the effects on cholesterol synthesis of normal, one week nephrotic, and two week nephrotic rat liver and kidney subcellular fractions, each adjusted to 5 mg protein per incubation.

The hepatic large granule fractions from all groups were potently inhibitory. Control and two week nephrotic large granule fractions respectively inhibited 97% and 96%, while the corresponding one week nephrotic fraction inhibited 67%. All other hepatic subcellular fractions had only relatively minor effects on cholesterol synthesis.

As in liver the renal large granule fraction was less inhibitory at one week (85%) than either the corresponding control (99%) or two week nephrotic (98%) fraction. In contrast to the relative lack of effect of hepatic small granule and smooth microsome fractions, comparable renal fractions inhibited more than 96%. Renal cell sap had only relatively minor effects.

Effects of Renal Microsomes and Their Derivatives

The assay of varying concentrations of renal smooth microsomes resulted in a log-dose response curve (Fig. 1). When renal microsomes were fractionated with the use of EDTA and ultracentrifugation in a discontinuous sucrose gradient (19), the inhibitory activity was associated with the microsomal membrances (Table II).

Sonication of kidney smooth microsomes caused the majority of the inhibitory activity to become nonsedimentable at 105,000 *g* for 60 min (Table III). This solubilized inhibitor was eluted in the void volume of a 2.5 x 20 cm

TABLE V

Effects of Intact and Sonicated Erythrocyte Ghosts on the In Vitro Incorproation of 2-^{14}C-Acetate Into Cholesterol by a Rat Liver Enzyme System

Experimental conditions[a]	DPM ± 1 S.D.
H_2O control	5370 ± 250
Intact erythrocyte ghosts, 2 mg protein	5040 ± 150
Sonicated erythrocyte ghosts, 2 mg protein	4680 ± 300

[a]Three incubations per group.

TABLE VI

Effects of Phospholipids on the Incorporation of 2-^{14}C-Acetate Into Cholesterol by a Rat Liver Enzyme System

Experimental conditions[a]	DPM ± 1 S.D.
Saline control	12500 ± 900
Phosphatidyl choline, 2 mg	12200 ± 590
Phosphatidyl ethanolamine, 2 mg	8870 ± 1300
Phosphatidyl inositol, 2 mg	3240 ± 260

[a]Three incubations per group.

TABLE VII

The Effects of Rat Kidney Lysosomal and Mitochondrial Hypotonic Extracts and Similarly Prepared Liver Extracts on the Incorporation of 2-^{14}C-Acetate Into Cholesterol by a Rat Liver Enzyme System

Experimental conditions[a]	DPM ± 1 S.D.
Phosphate buffer, 0.02 M, pH 6.8	7390 ± 1040
Kidney lysosomal extract, 0.8 mg protein	18400 ± 2040
Kidney mitochondrial extract, 1.3 mg protein	3420 ± 1520
Liver lysosomal extract, 1.9 mg protein	1190 ± 260
Liver mitochondrial extract, 2.3 mg protein	1300 ± 380

[a] Four incubations per group.

TABLE VIII

Effects of Rat Kidney Lysosomal Extract on the Incorporation of 2-^{14}C-Acetate and 2-^{14}C-Mevalonate Into Cholesterol by a Rat Liver Enzyme System

Experimental conditions	Substrate	DPM ± 1 S.D.
Phosphate control[a]	Acetate	46100 ± 3720
Kidney lysosomal extract,[b] 2 mg protein	Acetate	143000 ± 32800
Phosphate control[b]	Mevalonate	199000 ± 4610
Kidney lysosomal extract,[b] 2 mg protein	Mevalonate	192000 ± 5760

[a]Three incubations per group.
[b]Two incubations per group.

column containing BioGel P-150, suggesting a molecular weight in excess of 150,000. Renal microsomes were strongly inhibitory toward cholesterol synthesis from acetate but only mildly inhibitory from mevalonate (Table IV). The inhibitor is stable indefinitely at -75 C and at 4 C for one week.

Neither intact nor sonicated rat erythrocyte membranes prepared by the method of Dodge et al. (20) were inhibitory at a dose of 2 mg protein per incubation (Table V). Phospholipids in the amount of 2 mg per incubation were also tested. Phosphatidyl choline did not inhibit at this level while phosphatidyl ethanolamine inhibited 29% and phosphatidyl inisitol inhibited 74% (Table VI).

Effect of Hypotonic Extracts of Liver and Kidney Fractions and Related Substances

The effects of hypotonic extracts of purified liver and kidney mitochondrial and lysosomal fractions are presented in Table VII. All fractions were inhibitory except that derived from kidney lysosomes. The kidney lysosomal extract adjusted to 0.8 mg of protein per incubation more than doubled (+150%) the rate of incorporation of 2-^{14}C-acetate into cholesterol. While the lysosomal extracts regularly stimulate in vitro cholesterol synthesis from acetate, they show no significant effects when mevalonate is used as substrate (Table VIII). This stimulatory factor was eluted in the void volume of a 2.5 x 20 cm column containing Bio Gel P-150, suggesting a molecular weight in excess of 150,000. It was stable for several months at -75 C but unstable at 4 C. Two enzymes with known effects similar to the enzymes of renal lysosomes were tested in vitro. One milligram of trypsin abolished cholesterol synthesis while egg white lysozyme had no effect (Table IX).

Effects of Serum Proteins and Nonprotein Polymers on Cholesterol Synthesis

Purified bovine serum protein fractions adjusted to 5 mg per incubation were mildly inhibitory toward cholesterol synthesis (Table X). Albumin and α-globulin were more inhibitory than β-globulin and γ-globulin. In contrast the nonprotein polymers polyvinylpyrrolidone, dextran-40, dextran-80 and dextran-150 had no effect on the in vitro incorporation of acetate into cholesterol.

DISCUSSION

Albumin and other serum proteins of bovine origin are only mildly inhibitory toward hepatic cholesterol synthesis. Although the mechanism of this inhibitory effect is unclear, it is not related to oncotic pressure since nonprotein polymers have no effect. While the importance of the mild inhibitory effects of serum proteins remains to be determined, a specific relationship between hypoalbuminemia and hyperlipemia, as proposed by Marsh and Drabkin (3), appears unlikely. It is perhaps more likely that the kidney influences lipid metabolism by the production of agents which either stimulate or inhibit hepatic lipid synthesis as first proposed by Heymann and Hackel (5).

The large granule fractions of both liver and kidney are markedly inhibitory toward cholesterol synthesis, presumably because of the presence of the mitrochondrial inhibitor of cholesterol synthesis previously reported in liver tissue by Migicovsky (21). The renal fractions with the most significantly different effects from the corresponding hepatic frac-

TABLE IX

Effects of Trypsin and Egg White Lysozyme on the In Vitro Incorporation of 2-^{14}C-Acetate Into Cholesterol by a Rat Liver Enzyme System

Experimental conditions[a]	DPM ± 1 S.D.
Saline control	13200 ± 1470
Trypsin, 1 mg	21 ± 27
Egg white lysozyme, 1 mg	15900 ± 2730

[a]Three incubations per group.

TABLE X

The Effects of Bovine Serum Protein Fractions on the Incorporation of 2-^{14}C-Acetate Into Cholesterol by a Rat Liver Enzyme System

Experimental conditions[a]	DPM ± 1 S.D.
Saline control	7810 ± 680
Bovine serum albumin, 5 mg	5440[b] ± 320
Bovine serum α-globulin, 5 mg	5590[b] ± 490
Bovine serum β-globulin, 5 mg	6320[c] ± 720
Bovine serum γ-globulin, 5 mg	6670[c] ± 470

[a]Four incubations per group.
[b]$P < 0.01$.
[c]$P < 0.05$.

tions are those which contain microsomes, that is the small granule and smooth microsome fractions (10). The inhibitory material is present in highest concentration in the membranes of the endoplasmic reticulum. This high molecular weight inhibitor has primarily a pre-mevalonate site of inhibition in the biosynthesis of cholesterol. This inhibition is not a property of all trilaminar membranes since rat erythrocyte membranes have no effect. Similarly the inhibition does not appear to be due to the phospholipid content of the renal microsomal membranes since lecithin, the predominant phospholipid of most membranes (22), is not inhibitory. Phosphatidyl ethanolamine and phosphatidyl inositol are inhibitory. However because of their low concentrations in microsomal membranes, the inhibitory activity of renal microsomes cannot be ascribed to their phospholipid content. Further since the phospholipid content of rat kidney and liver smooth microsomes would not be expected to differ greatly (23), the inhibitory activity of renal smooth microsomes is most likely associated with the protein of the renal endoplasmic reticulum.

Several theories can be offered to explain the potent inhibitory effect of renal microsome-containing fractions while comparable hepatic fractions lack inhibitory effects. Possibly the microsome-containing fractions from liver and kidney contain different organelles. However the fractions from the two tissues were similarly isolated and all of the subcellular particles were retained using an arbitrary fractionation scheme. Rat kidney microsomal fractions have been reported to contain appreciable amounts of microvilli from renal tubular cells (24). However such microvilli-containing fractions were separated following vigorous Waring blender homogenization, a technique known to disrupt many cellular membranes (9). In contrast membrane-containing fractions in the present experiments were prepared after gentle Dounce homogenization. Nevertheless even if the inhibitory activity is associated with fragments of specialized renal plasma membranes, an inhibitor in this location would be of interest.

Since renal microsomes contain a peptidase active at the pH of the assay system (25), inhibition might be attributed to proteolysis. This is a plausible explanation since trypsin abolished cholesterol synthesis, presumably by hydrolyzing necessary enzymes. However the activity of this specific renal peptidase is enhanced in tris buffer (25). Since additive effects of tris buffer and antilipemic factor were not noted, the inhibitory activity cannot be due to this protease.

The moderate hyperlipemia which accompanies nephrectomy and toxic nephroses and Lagrue's observation that normal kidney tissue prevents nephrotic hyperlipemia (7) strongly suggest that kidneys normally produce a factor capable of inhibiting cholesterol biosynthesis in vivo. Conceivably the microsomal antilepemic factor reported in this study is identical with the renal factor. Further purification of this inhibitor and in vivo testing will be necessary before this conclusion can be accepted.

The observed stimulatory principle within renal lysosomes fulfills the hypothesis of Heymann and Hackel (5) and Tracy (6). This pre-mevalonate stimulator of cholesterol synthesis is present within a renal subcellular fraction which has been described as originating from the phagolysosomes (10). During nephrosis, enhanced renal protein reabsorption occurs with marked increases in the number and activity of renal lysosomes.

A potential route of entry into the liver of the high molecular weight renal lipemic and antilipemic factors is via hepatic lysosomes. Hepatic lysosomes increase in number as early as four days after Aminonucleoside injection (26), and they are known to remove certain macromolecules from blood. The degree of hepatic lysosomal uptake is proportional to the molecular weight of the macromolecule (27). Thus the high molecular weight of these renal macromolecules would actually favor uptake by hepatic lysosomes. Evidence that the kidneys affect the liver's content of specific proteins is provided by the regular occurrence of hepatic enzyme changes during nephrosis (28,29), as well as in non-nephrotic proteinuria (30). Further, hepatic lysosomal enzymes are increased in patients with nonmetastatic hypernephromas, possibly because of uptake by hepatic lysosomes of enzymes produced by the renal tumor (31).

The renal lipemic and antilipemic factors reported here are compatible with the effectors postulated by Heymann and Hackel (5), Tracy (6), and Lagrue et al. (7) based on their work with intact animals. However since the effects of the crude lipemic and antilipemic factors have been demonstrated only in vitro in cell free systems, further purification of these factors and testing in whole cell systems and in intact animals is required before conclusions can be reached regarding possible physiologic effects of such factors.

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Volatile Compounds From Thermally Oxidized Methyl Oleate[1]

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ABSTRACT

Thermal oxidation of methyl oleate was studied over a range of temperatures from 50 C to 150 C for periods of time up to 30 min. Degradation was quantitatively followed by gas liquid chromatography (GLC) and liquid scintillation counting of the products of methyl oleate-U-^{14}C heated under a stream of compressed air. Heptane, octane, 2-decanone, benzene, *o*-xylene, methyl hexanoate, methyl heptanoate and methyl octanoate were identified by GLC and mass spectrometry. Mass spectral evidence also was obtained for methyl pimelaldehydate, methyl suberaldehydate and methyl azelaaldehydate. Organic synthesis confirmed the identity of methyl azelaaldehydate. Most of the products formed suggested that autoxidation was responsible for the degradation occurring at the temperatures employed in this study.

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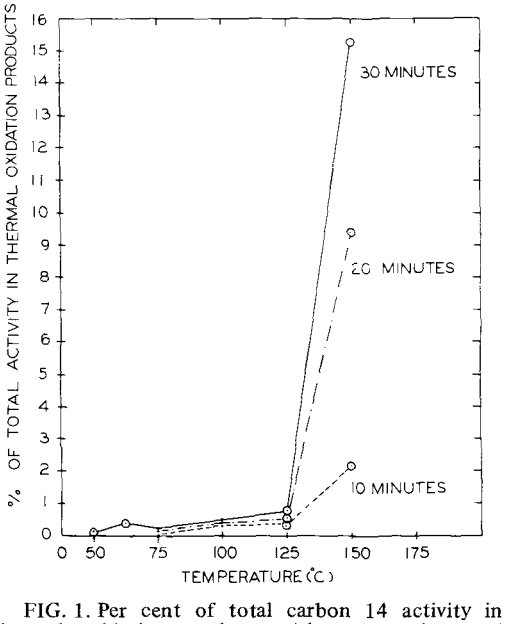


FIG. 1. Per cent of total carbon 14 activity in thermal oxidation products with various time and temperature treatments.

INTRODUCTION

Much of the previous work concerning the degradation of lipids, fatty acids and methyl esters of fatty acids has dealt with autoxidation and its products. The mechanism of autoxidation has been reviewed by several investigators (3,4,10,16), yet no single theory receives total acceptance (8). Carbonyl compounds resulting from the decomposition of the hydroperoxides are important constituents of oxidized fats (4). Flavor and odor thresholds for many of these carbonyls are at levels less than 1 ppm and are important to the sensory qualities of numerous foods (14). Volatile decomposition products of methyl oleate resulting from open air heating at 200 C were examined by Toi et al. (17). Their results showed the presence of C_8 and C_9 aldehydes, semialdehyde methyl esters, C_7 and C_8 hydrocarbons, methyl esters of fatty acids, several fatty acids, mono-methyl esters of dibasic acids and some alcohols.

The objective of this investigation was to evaluate quantitatively and qualitatively the thermal oxidation of methyl oleate heated at various temperatures. Experimental conditions were less extreme than those previously reported by Toi et al. (17), and gas chromatography coupled with mass spectrometry was employed.

EXPERIMENTAL PROCEDURE

Thermal Oxidation of Methyl Oleate

A glass system included a 10 ml Bantamware (Kontes) pear-shaped flask connected to a Bantamware reflux condenser and drying tube. A 1/16 in. Teflon tube was attached to a glass capillary tube that extended to the bottom of the reaction flask. The Teflon tube was connected to a tank of compressed breathing air that purged the sample at a rate of 30 ml/min.

Samples were prepared by adding 10 μl of methyl oleate-U-^{14}C (uniformly labeled), prepared by the procedure of Metcalfe and Schmitz (12) from oleic acid U-^{14}C (sp. act. 630 mc/mM, 99+% radio purity) to 250 μl of unlabeled methyl oleate (99+% purity). Chemicals for this aspect of the study were obtained

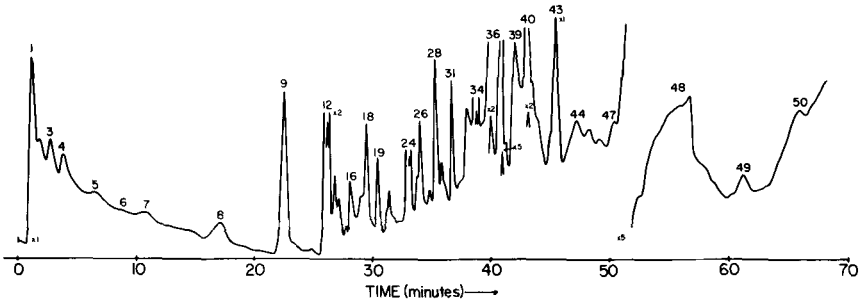


FIG. 2. Gas chromatogram of the volatile compounds from thermally oxidized methyl oleate.

from Applied Science Laboratories. After preparation each sample was subjected to one of the following heat treatments (±0.01 C): 50 C for 30 min; 62 C for 30 min; 75 C for 10, 20 or 30 min; 100 C for 10, 20 or 30 min; 125 C for 10, 20 or 30 min; or 150 C for 10, 20 or 30 min. After heat treatment the samples were stored in tightly-stoppered vials at 5 C until analyzed by gas liquid chromatography (GLC) and liquid scintillation counting.

Samples were analyzed with a modified Aerograph Model 90-P3 gas chromatograph that employed dual 12 ft x 1/8 in. OD stainless steel columns packed with 5% DEGS (diethylene glycol succinate) on Chromosorb G, a Carle Model 100 ultramicro bead detector system, and a Micro-Tek Model GC-2500 linear temperature programmer. These modifications improved the sensitivity of the GLC analysis while allowing the total effluent to be trapped by a Packard Tri-Carb Model 830 gas chromatograph fraction collector modified for subambient temperature operation. Glass cartridges filled with anthracene crystals coated with silicone oil, described by Karmen et al. (7), were used for trapping. Duplicate counts of the trapped fractions were obtained with a Nuclear-Chicago Model 6766 liquid scintillation counter. Each sample was counted to a 1% relative standard deviation (18). The activity of each fraction was calculated as a percentage of the total activity trapped in all fractions of a particular GLC analysis.

Identification of Volatile Compounds

Unlabeled methyl oleate heated at 150 C for 90 min, in the system described above, was used for product identification. The extended heat treatment increased the concentration of components of interest that appeared in the labeled compound experiments. Volatile compounds were analyzed by a combined GLC-mass spectrometer system. An F&M Model 810 gas chromatograph was fitted with a 300 ft x

TABLE I

GLC[a] and Mass Spectral Identification of Some Compounds of Thermally Oxidized Methyl Oleate

Compound	Peak no.	Relative retention time[b]		Mass spectra ref.
		Heated methyl oleate	Authentic	
Heptane	3	0.049	0.050	1
Octane	4	0.065	0.061	1
Benzene	5	0.115	0.117	1
Methyl hexanoate	6	0.362	0.358	2
o-Xylene	---[c]	0.372	0.343	1
Methyl heptanoate	7	0.593	0.595	2
Methyl octanoate	9	1.000	1.000	2
2-Decanone	24	1.406	1.400	2
Methyl pimelaldehydate	43	2.049	---	---
Methyl suberaldehydate	47	2.230	---	---
Methyl azelaaldehydate	48	2.412	2.434	19

[a]GLC, gas liquid chromatography.

[b]Relative to methyl octanoate. 12 ft x 1/8 in. OD stainless steel column packed with 5% DEGS on Chromosorb G. Temperature programmed at 4 C/min from 90-190 C with 15 min initial hold.

[c]Gas chromatographic peak not observed.

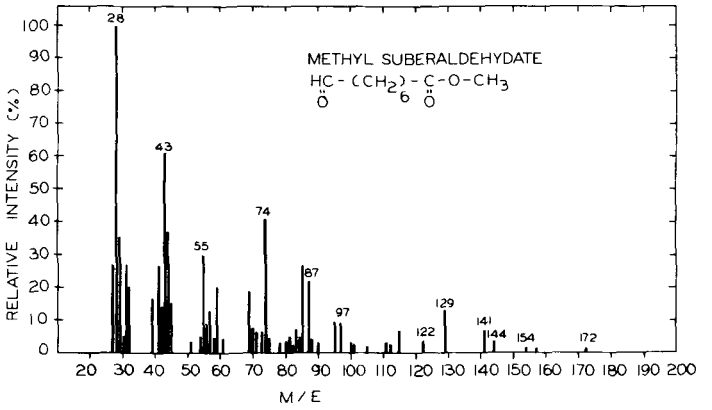


FIG. 3. Mass spectrum of compound tentatively identified as methyl suberaldehydate.

0.01 in. ID Golay type capillary column with butanediol succinate (BDS) as the stationary liquid phase. Operating conditions for this inlet system were 180 C isothermal column temperature with a flow rate of 1 ml per min of helium.

The Golay column was directly connected to the double ion source of an Atlas CH-4 mass spectrometer; the high vacuum was 1.5 x 10^{-6} mm of Hg while admitting GLC effluent. The double ion source gave both mass spectral oscillograms and a strip chart gas chromatogram.

GLC effluent fractions were also collected from a 12 ft x 1/8 in. OD stainless steel column containing 5% DEGS on Chromosorb G, rechromatographed and recollected from a 12 ft x 1/8 in. OD stainless steel column containing 3% BDS on Chromosorb G, and examined with the static reservoir inlet system of the Atlas CH-4. This technique enabled low voltage studies and also allowed the accurate measurement of the parent $(P)^+$, $(P+1)^+$ and $(P+2)^+$ ion intensities which are often helpful in assigning an empirical formula.

A third mass spectrometer inlet system utilized the EC-1 gas inlet valve which permitted regulation of the amount of GLC packed column effluent admitted to the double ion source of the Atlas CH-4. The column used with this inlet system was a 12 ft x 1/8 in. OD stainless steel column containing 5% DEGS on Chromosorb G. Operating conditions were a flow rate of 25 ml of helium per min and a temperature program from 90-190 C at 4 C per min after a 15 min initial hold.

Methyl azelaaldehydate was synthesized from methyl oleate via hydroxylation with osmium tetroxide and subsequent oxidative cleavage with lead tetra-acetate. This synthesis yielded *n*-nonanal, nonanoic acid, and methyl azelaaldehydate which were easily separated by GLC and analyzed with the static reservoir inlet system of the Atlas CH-4. In retrospect the synthesis of methyl azelaaldehydate via ozonolysis and subsequent purification as the bisulfate compound appears to be a better route

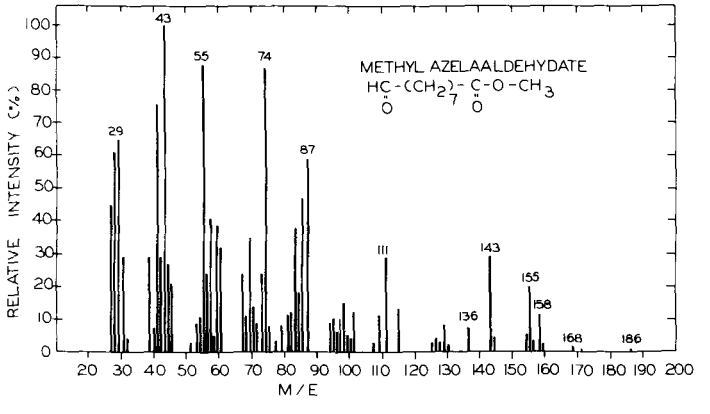


FIG. 4. Mass spectrum of authentic methyl azelaaldehydate.

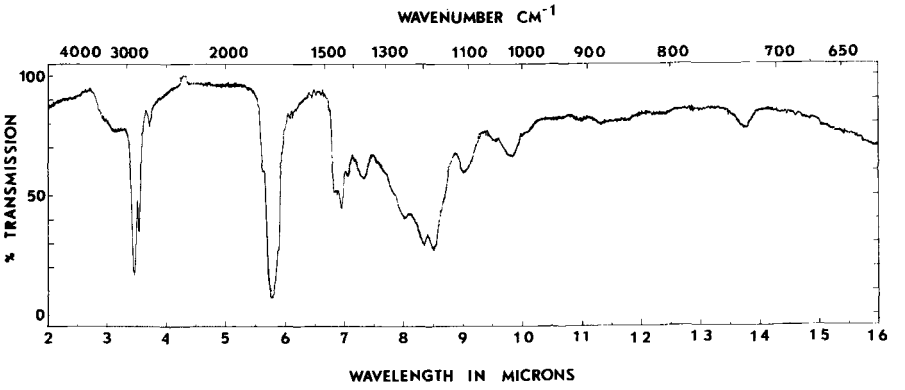


FIG. 5. IR spectrum of authentic methyl azelaaldehydate.

(13).

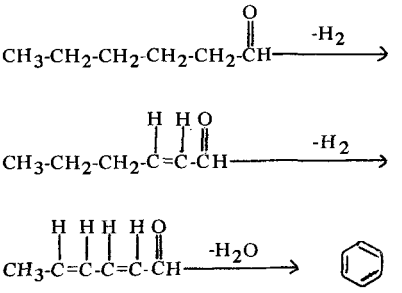
IR analyses were performed with a Beckman IR-5A spectrometer.

RESULTS AND DISCUSSION

Quantitative data in Figure 1 show the percentage of the total carbon 14 activity that appeared in GLC fractions other than the methyl oleate fraction. Of the total sample carbon 14 activity in the original sample, approximately 13% appeared in all volatile fractions including methyl oleate and the remaining 87% apparently remained in the polymeric fraction or free acid form. Thermal oxidation products accounted for less than 1% of the total activity through the 125 C treatments.

Significant production of oxidation products did not occur until the temperature of the reaction reached 150 C. The temperature of the treatment is a more important factor than time in the thermal oxidation process.

The gas chromatogram of the 150 C sample (Fig. 2) shows the presence of approximately 50 components from the thermally oxidized methyl oleate. Evidence for an additional 12 compounds was obtained in related chromatographic analyses. Some of the major volatile compounds were identified (Table I). The presence of the C_7 and C_8 *n*-alkanes has been reported in gamma-irradiated milk fat (9), gamma-irradiated methyl oleate (11), and in methyl oleate held at 200 C (17). These compounds appeared abundantly in the low (50 C, 30 min) and high (150 C, 30 min) temperature-treated systems of this study. Benzene was present in much smaller quantities than the *n*-alkanes and may be an artifact. Its presence was reported in gamma-irradiated milk fat (9) and in gamma-irradiated methyl oleate (11). Khatri (9) has indicated that aldehydes of six carbons or more tend to cyclize to aromatic compounds at high temperatures in the inlet system of the mass spectrometer. The aldehyde may possibly undergo a dehydrogenation and a subsequent dehydration reaction similar to the one shown below at elevated temperatures found in the inlet system of the mass spectrometer as well as in the model system reactions.



Fritsch and Deatherage (6) found the C_6, C_7 and C_8 saturated fatty acids as decomposition products of methyl oleate. Subsequently Toi et al. (17) found the C_6, C_7 and C_8 fatty acid methyl esters from heated methyl oleate. The presence of 2-decanone has previously been reported by Scanlan et al. (15) in heated milk and was present in the heated methyl oleate in limited concentrations relative to the amount of short chain fatty acids present. It has been well established that the odd numbered *n*-alkyl methyl ketones are produced from beta-keto acids; however the precursors and mechanisms of formation of the even-numbered *n*-methyl ketone have not been elucidated. The presence of the 2-decanone in this system suggests that an ester of oleic acid may serve as a precursor for its formation.

The semialdehyde methyl esters have been studied previously (4,5,17), but spectrometric data were available for only methyl azelaaldehydate (19). The mass spectrum of the fraction

tentatively identified as methyl suberaldehydate was obtained using the 70 ev ion source and the static reservoir system (Fig. 3).

Low voltage spectra at 11 ev helped establish the apparent molecular ion at m/e 172 and 0.6% relative intensity to the base peak. Peaks at m/e 74 (41%) and 87 (22%) were indicative of a methyl ester. The base peak at 28 (100%) along with the peak at 144 (4%), P-28, may result from the carbonyl moiety. Other characteristic peaks were 129 (12%), 141 (7%), 115 (7%) and 154 (1%). The mass spectrum of the fraction tentatively identified as methyl azelaaldehydate showed an apparent molecular ion at m/e 186 (0.01%) and a base peak at 28 (100%) with the associated peak at 158 (8%), P-28. Peaks at m/e 74 (88%) and 87 (63%) were again indicative of a methyl ester. Other characteristic peaks that were related to the methyl suberaldehydate by the mass difference of one methylene unit were 143 (21%), 155 (16%) and 129 (14%).

The mass spectrum of authentic methyl azelaaldehydate shown in Figure 4 is in agreement with the spectrum of the tentatively identified compound and also with the spectrum of authentic methyl azelaaldehydate obtained from the Northern Regional Research Laboratory, Peoria, Illinois. However the mass spectrum of methyl azelaaldehydate recently published by Yasuda et al. (19) is somewhat different. Certain ions, m/e 168 (P-18), 158 (P-28) and 155 (P-31) which we observed and found important in mass spectral interpretation, are absent in this previously published spectrum (19).

Figure 5 is the infrared spectrum of authentic methyl azelaaldehydate. There are strong CH stretch bands at 2700 and 2900 cm^{-1} and a strong aldehyde carbonyl stretch at 1740 cm^{-1}.

The infrared spectra of methyl suberaldehydate and methyl azelaaldehydate from thermally oxidized methyl oleate showed characteristic fingerprint bands similar to the authentic methyl azelaaldehydate. Mass spectral evidence also suggested the presence of the next lower member of the homologous series, methyl pimelaldehydate. A linear relationship was observed in a plot of relative retention times versus carbon number for the three semialdehyde homologs. The observed odor of the semialdehyde fractions was suggestive of the objectionable oxidized odor of cooking fats.

In conclusion at least 62 compounds were separated from heated methyl oleate. Mass spectral evidence has been given for several volatile products, the major component being methyl azelaaldehydate. Two other members of the homologous series of semialdehyde methyl esters were tentatively identified and each appeared to have important sensory qualities in heated fats.

ACKNOWLEDGMENTS

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Wax Esters in the Cystacanths of *Polymorphus minutus* (Acanthocephala)

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ABSTRACT

The lipids of the cystacanth of the acanthocephalan *Polymorphus minutus* have been analyzed. Wax esters constituted nearly 90% of the total cystacanth lipids. The wax ester fraction contained approximately 10% steroid ester; the rest was long chain alcohols C_{12} to C_{20}, largely saturated, esterified with fatty acids C_{12} to C_{22}, mostly unsaturated, with C_{18} predominating. Corresponding quantities of wax esters were not found in the adult parasite. Cholesterol was identified as the only steroid present in the cystacanth.

INTRODUCTION

Polymorphus minutus is an acanthocephalan parasite the adults of which infect the intestines of water fowl, while the juvenile stages develop in amphipods of the genus *Gammarus*. The fully developed juvenile in the haemocoel of the *Gammarus* is called a cystacanth. This is a resting stage and when the infected *Gammarus* is eaten by a duck, the cystacanth is released and develops into an adult parasite in the host's intestine.

There have been few studies on the lipids of adult Acanthocephala (1,2) and none at all on the lipids of the juvenile stages. In this paper the lipid composition of the cystacanth of *Polymorphus minutus* is described and was found to contain a high proportion of wax esters, a lipid fraction not present in the adult parasite.

EXPERIMENTAL PROCEDURES

Preparation of Material

Cystacanths of *P. minutus* were obtained from naturally infected *Gammarus pulex*. The lipids were extracted by the method of Folch et al. (3); total lipid content was determined gravimetrically using an electro-microbalance (Research and Industrial Instruments EMB-1).

Thin Layer Chromatography

Quantitative analysis of the major lipid fractions was carried out by semi-micro thin layer chromatography (TLC) on plates (7.5 x 2.5 cm) coated with Silica Gel G (250 μm thick). The neutral lipids were fractionated with hexane-diethyl ether-ethyl acetate-acetic acid (90:5:3:2 v/v) and the phospholipids with chloroform-methanol-water-acetic acid (60:15:3:2 v/v). The lipids were charred at 200 C after treatment with sulfuryl chloride vapor (4) and the position and optical density of the spots measured with a Joyce Loebl Chromoscan. This method is quantitative (5,6), the mean coefficient of variation of the method being 4.3% for the neutral lipids and 6.5% for the phospholipids. Calibration curves were prepared for the different lipid classes using lipid standards obtained from Sigma Ltd.

Analytical and preparative TLC was performed on 8.25 x 12 cm plates coated with Silica Gel G (250 μm thick). The neutral lipids were separated by a double development sys-

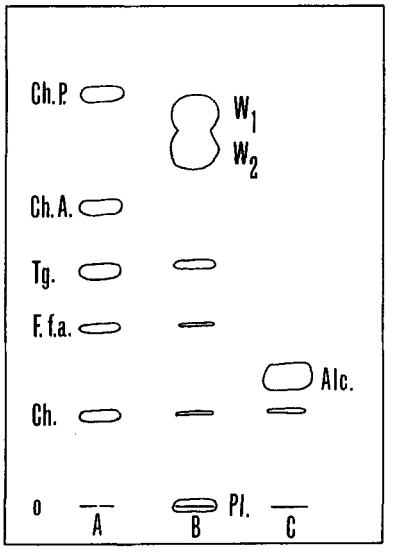

FIG. 1. Thin layer plate of *Polymorphus minutus* cystacanth neutral lipids, solvent 1, hexane-diethyl ether-acetic acid-ethyl acetate (30:7:1:2) run two thirds of the way up the plate; Solvent 2, hexane-diethyl ether (30:2) to the top of the plate. A, Lipid standards; B, cystacanth lipids; C, neutral nonsaponifiables from the wax esters. Alc., long chain alcohols; Ch., cholesterol; Ch A., cholesterol acetate; Ch. P., cholesterol palmitate; F.f.a., free fatty acids; o, origin; Pl., phospholipids; Tg., triglycerides; W_1 and W_2, wax 1 and wax 2.

TABLE I

The Lipids of the Cystacanth and Adult *Polymorphus minutus*

	Weight % of total lipids (mean ± S.E. n=4)	
	Cystacanth	Adult
Neutral lipids	93.0 ± 3.9	59.2 ± 3.8
Diglycerides	Trace[a]	Trace
Steroids	0.6 ± 0.02	13.4 ± 0.6
Free fatty acids	1.0 ± 0.04	7.2 ± 0.4
Triglycerides	2.4 ± 0.1	23.8 ± 1.0
Wax and sterol esters	89.0 ± 4.3	14.8 ± 0.6
Phospholipids	7.0 ± 0.45	40.8 ± 2.7
Phosphatidyl serine	Trace	Trace
Lyso-phosphatidyl choline	Trace	0.5 ± 0.03
Sphingomyelin	Trace	1.6 ± 0.1
Phosphatidyl choline	6.2 ± 0.3	31.5 ± 2.0
Phosphatidyl ethanolamine	0.8 ± 0.05	7.2 ± 0.5

[a]Trace = less than 0.25%

tem; solvent 1, hexane-diethyl ether-acetic acid-ethyl acetate (30:7:1:2 v/v) was run two thirds of the way up the plate; the plate was then dried and developed the whole way with solvent 2, hexane-diethyl ether (30:2 v/v). Phospholipids were separated using chloroform-methanol-ammonia (35% w/w aqueous) (65:25:5 v/v) and chloroform-acetone-methanol-acetic acid-water (3:4:1:1:0.5 v/v)(7).

Lipids were located by charring after spraying with 50% aqueous sulfuric acid (v/v) saturated with potassium dichromate (8). The lipids were identified by a comparison with standards obtained from Sigma Ltd. Specific spray reagents were also employed to aid identification of the various lipid fractions. Steroids and steroid esters were located using the ferric chloride reagent of Lowry (9); phospholipids were identified with an ammonium molybdate reagent (10); Dragenorf reagent (11) was used to locate choline compounds; lipids containing sugars were identified with α-napthol (12); and ninhydrin (7) was used to detect amino phosphatides.

For preparative TLC the lipids were located under UV light after spraying with aqueous rhodamine 6G (0.005%) or directly after spraying with 1% iodine in methanol. The bands were scraped from the plates and extracted into diethyl ether.

Wax esters isolated by TLC were saponified by refluxing with 2 N KOH in 70% methanol for several hours under nitrogen (13,14). The methanol was boiled off and the neutral nonsaponifiables (steroids and long chain alcohols) extracted with diethyl ether. The aqueous layer was then acidified with 12 N sulfuric acid and the free acids extracted into diethyl ether. Both extracts were washed repeatedly with water and then dried over anhydrous sodium sulfate. The neutral nonsaponifiables were fractionated by preparative TLC into long chain alcohols and steroids.

Gas Liquid Chromatography

A Pye series 104 gas chromatograph equipped with a flame ionization detector and 1.5 m coiled glass columns was used. Methyl esters of the fatty acids of the various lipid fractions were prepared using the boron trifluoride-methanol reagent (15). The methyl esters of the fatty acids were separated on 15% polyethylene glycol adipate (PEGA) on EMBALAL 60-100 mesh, nitrogen flow rate 70 ml/min, operated either isothermally at 175 C (to calculate carbon numbers) or else programmed from 150-210 C at 4 C/min. The fatty acid methyl esters were also analyzed on 3% SE-30 on CQ, 100-120 mesh, nitrogen flow rate 80 ml/min, under the same conditions as above. The relative amounts of the fatty acids were calculated from the peak areas (16). Standard methyl esters were obtained from Sigma Ltd. and the fatty acids were identified by their relative retention times on the different columns.

Steroids were analyzed both as free steroids and as their trimethylsilyl (TMS) derivatives (17,18), on 3% SE-30 on CQ 100-120 mesh, nitrogen flow rate 80 ml/min at 275 C and on 3% QF-1 on CQ 100-120 mesh, nitrogen flow rate 100 ml/min at 245 C. The steroids were identified by comparison of their relative retention times on the two columns with known standards.

The long chain alcohols were analyzed as free alcohols on 3% SE-30 on CQ 100-120 mesh nitrogen flow rate 70 ml/min either isothermally at 175 C or programmed from 125-200 C

TABLE II

Long Chain Alcohol Composition of the Wax Esters From the Cystacanths of *Polymorphus minutus*

Alcohol	Carbon[a] No.	Weight % (mean ± S.E. n=5) Wax 1	Wax 2
12:0	12	Trace[b]	---
14:0	14	1.3 ± 0.3	Trace
15:0	15	Trace	---
16:0	16	48.4 ± 2.7	17.6 ± 2.4
17:0	17	4.9 ± 1.0	---±
18:0	18	39.8 ± 3.9	39.2 ± 5.0
18:1	18.6	3.0 ± 1.0	10.0 ± 1.3
18:2	19.4	Trace	Trace
20:0	20	2.6 ± 0.7	33.2 ± 3.4
20:1	20.5	Trace	Trace

[a]on 3% SE-30

[b]Trace = less than 0.5%

at 1.5 C/min. The TMS derivatives of the alcohols were prepared (17) and separated on 3% QF-1 on CQ 100-120 mesh, nitrogen flow rate 70 ml/min at 165 C or on 15% PEGA on EMBALAL 60-100 mesh, nitrogen flow rate 70 ml/min at 175 C.

Authentic long chain alcohols were synthesized by reduction of the corresponding fatty acid methyl esters with lithium aluminum hydride in dry ether (19). After the reduction the alcohols were purified by preparative TLC. The following alcohols were synthesized: saturated, even carbon numbers from C_8 to C_{22} and unsaturated $C_{16:1}$, $C_{18:1}$, $C_{18:2}$, $C_{18:3}$. Alcohols for which there were no standards were identified from their carbon numbers.

Standard errors were calculated by the methods of Dean and Dixon (20).

Infrared Spectroscopy

IR absorption spectra were obtained with a Unicam SP 200 G IR spectrophotmeter using silver chloride microcells, pathlength 0.01 mm. (Research and Industrial Instrument Co.). The samples were dissolved in diethyl ether or carbon tetrachloride.

RESULTS

Lipid constituted 28.2 ± 3 (mean ± S.D. n = 4)% of the dry weight of the cystacanths of

TABLE III

Component Fatty Acids of the Lipids From the Cystacanths of *Polymorphus minutus*

Fatty acid	Carbon[a] No.	Weight % (mean ± S.E. n=5) Wax 1	Wax 2	Triglycerides	Free fatty acids	Phospholipids
8:0	8	---	---	Trace[b]	---	---
10:0	10	---	---	Trace	---	Trace
12:0	12	3.25 ±0.75	0.75 ±0.5	1.1 ±0.64	Trace	Trace
12:1	12.3	---	---	Trace	---	Trace
14:0	14	6.0 ±1.0	3.0 ±0.6	2.25 ±1.0	1.0 ±0.25	0.25 ±0.1
14:1	14.3	---	---	Trace	---	Trace
16:0	16	30.0 ±3.3	27.4 ±3.3	35.0 ±5.0	11.4 ±1.3	32.85 ±2.9
16:1	16.2	3.0 ±0.5	Trace	4.5 ±0.5	---	4.4 ±0.55
17:0	17	Trace	---	---	----	---
18:0	18	8.0 ±1.5	5.25 ±1.0	8.7 ±1.1	57.1 ±2.0	7.1 ±3.0
18:1	18.2	38.25 ±4.0	50.1 ±2.6	42.8 ±5.8	30.5 ±2.6	46.3 ±2.2
18:2	18.8	4.0 ±1.3	5.0 ±1.5	5.4 ±1.3	---	5.9 ±1.1
18:3	19.6	2.0 ±0.3	7.0 ±2.0	---	---	2.2 ±0.4
18:4	20.3	Trace	---	---	---	---
20:2	20.6	3.0 ±0.5	---	0.25 ±0.12	---	---
20:3	21.2	2.0 ±0.5	---	---	---	---
20:4	22.4	---	---	---	---	1.0 ±0.2
22:2	22.8	0.5 ±0.3	1.5 ±0.3	---	---	---

[a]on 12% PEGA

[b]Trace = less than 0.1%

P. minutus, the dry weight of a mature cystacanth of *P. minutus* being 0.110 ± 0.01 (mean ± S.D. $n = 5$)mg. The lipids from the cystacanths were separated into the major lipid classes by TLC (Fig. 1) and the results are shown in Table I, together with the lipid analysis of adult *P. minutus*, for comparison.

The major lipid component from the cystacanths consisted of a mixture of wax and sterol esters which constituted nearly 90% of the total lipid. These esters could be resolved by TLC into two overlapping spots (wax 1 and 2). The IR spectrum of the wax esters showed absorption peaks at 1070, 1260 and 1755 cm^{-1}, characteristic of aliphatic esters. Hydrolysis of the wax esters yielded free fatty acids, long chain alcohols and steroids. The steroids constituted approximately 10% of the total esters and gas liquid chromatography (GLC) of the isolated steroids yielded only a single peak which was identified as cholesterol. Insufficient material was available for a satisfactory IR spectrum of the cystacanth steroid, but the spectrum obtained was consistent with the compound being cholesterol.

IR spectra of the long chain alcohols from the wax esters showed absorption peaks at 1025 cm^{-1}, a weak peak at 1260 cm^{-1} and a broad peak centered on 3000 cm^{-1}, confirming that they were primary alcohols. The long chain alcohols were characterized by GLC and the results are shown in Table II.

The fatty acid composition of the waxes and of the major lipid fractions was determined by GLC and the results are summarized in Table III.

The free steroids were analyzed by GLC and as with the esterified steroids only a single peak was found which corresponded to cholesterol.

DISCUSSION

The cystacanth is an infective resting stage in the life cycle of the parasite and like the corresponding infective stages of many other parasites contains large quantities of lipids. However the cystacanth of *P. minutus* is extremely unusual in storing large quantities of wax esters rather than the more usual triglycerides. Moreover wax esters are not present in any significant amounts in the adult parasite. Wax esters occur as a storage lipid in the protozoan *Euglena gracilis* (21,22) and in a number of marine organisms (23), particularly in marine copepods where they may act as a reserve energy store (24,25). The cystacanth of *P. minutus* is, however, the first record of wax esters occurring as a storage lipid in a parasitic invertebrate.

The significance of wax esters as a storage lipid rather than triglyceride is obscure. In marine organisms wax esters may have a buoyancy function since wax esters have a lower density than the corresponding triglycerides. Wax esters also have a lower melting point than the corresponding triglycerides and this may be important in the cystacanths of *P. minutus*. In the *Gammarus* the ambient temperature is in the range 4-17 C (26). At these low temperatures the wax esters still form a very fluid lipid and this may be involved in the hydrostatic system of the lacunar canals (27). When the cystacanths are eaten by the final host, a water fowl, there is a big increase in the ambient temperature. Changing from wax esters to triglycerides might enable the parasite to raise the melting point of its lipids without any drastic change in the fatty acid composition.

The only steroid found in the cystacanths of *P. minutus* was cholesterol. Cholesterol was also the major steroid found in the other acanthocephalans so far investigated, *Macracanthorhynchus hirudinaceus* and *Moniliformis dubius* (1,2), although these two species also contained small amounts of phytosterols.

The fatty acid composition of the cystacanths was not usual and was similar to the fatty acid composition reported for the two species of adult Acanthocephala, *M. dubius* and *M. hirudinaceus* (1). While the fatty acid moiety of the wax esters from the cystacanths of *P. minutus* is largely unsaturated, the alcohols are almost entirely saturated.

The origin of the wax esters in the cystacanths of *P. minutus* is unknown. Wax esters only occur in small amounts in the *Gammarus* (26); however the cystacanths of *P. minutus* may accumulate them preferentially in the same way as it preferentially accumulates certain carotenoids (28). Alternatively the wax esters may be synthesized de novo by the parasite. A comparative study of the reserve lipids of acanthocephalan cystacanths from crustacean and insect hosts might well reveal interesting results.

ACKNOWLEDGMENT

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The Structural Analysis of Wheat Flour Glycerolipids

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ABSTRACT

The compositions of the fatty acids in the 1, 2 and 3 positions of the principal glycerolipids and their various stereoisomers were determined. Fatty acids in the 1 and 3 positions of triglycerides were similar in composition and less unsaturated than those in the 2 position. Fatty acids in the 1,2-, 1,3- and 2,3-diglycerides were distributed in a pattern which indicated isomerization of *sn*-1,2-diacylglycerol. Lysophosphatidyl choline (the principal monoacyl lipid) consisted of about 80% 1-acyl and 20% 2-acyl isomers. The fatty acid compositions indicated that most of the 2-lysophosphatidyl choline was formed by isomerization of 1-lysophosphatidyl choline. Most of the diglycerides and lysophosphatidyl choline were synthesized in the ripening wheat grain. However a small proportion of these partial glycerides and all of the other minor partial glycerides (monoglycerides, digalactosyl monoglycerides) appeared to be the result of limited lipolysis of the corresponding diacyl lipids in the wheat or in the freshly-milled flour. Fatty acids in the 2 position of all the fully acylated glycerides were very similar in composition, but there were considerable differences in the 1 position fatty acids. The glyceride distributions in the diacyl glycerides correspond to 1 random, 2 random fatty acid distributions.

INTRODUCTION

A detailed quantitative analysis of 23 classes of lipids in a sample of flour, and methods for the separation of these lipids by thin layer chromatography (TLC) have been published (1,2). It has been shown that enzymic hydrolysis of glycerides occurs during the storage of cereals and flours (3,4), and enzymic oxidation of free fatty acids and monoglycerides occurs during aerobic dough mixing (5-7). These degradations may be stages in the breakdown of lipids occurring during germination. On the other hand, since the organized cell structure of the wheat grain has been seriously disturbed in flour milling, and since contamination from bacteria and molds has been introduced, these changes may be very different from those which take place during germination.

This paper deals with the structural composition of the principal glycerolipids in wheat flour lipids from the time the grain is harvested.

EXPERIMENTAL PROCEDURES

A freshly-milled commercial sample of unbleached, untreated high grade winter wheat flour was stored at -20 C. Under these conditions there were negligible changes in the lipids over a period of a year.

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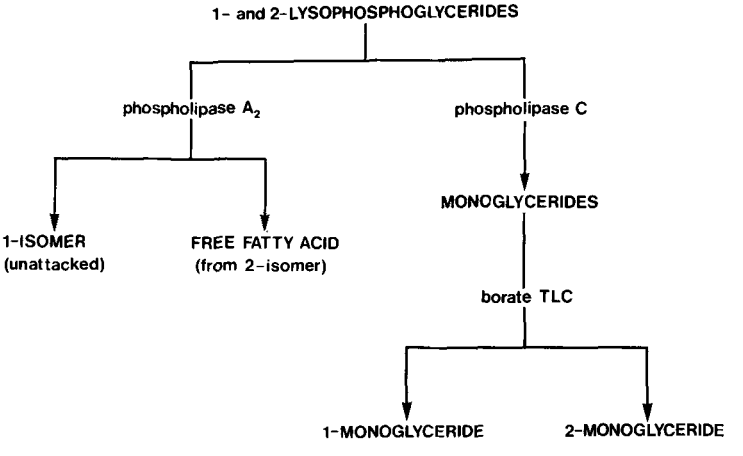

FIG. 1. Scheme for the analysis of monoacyl glycerides.

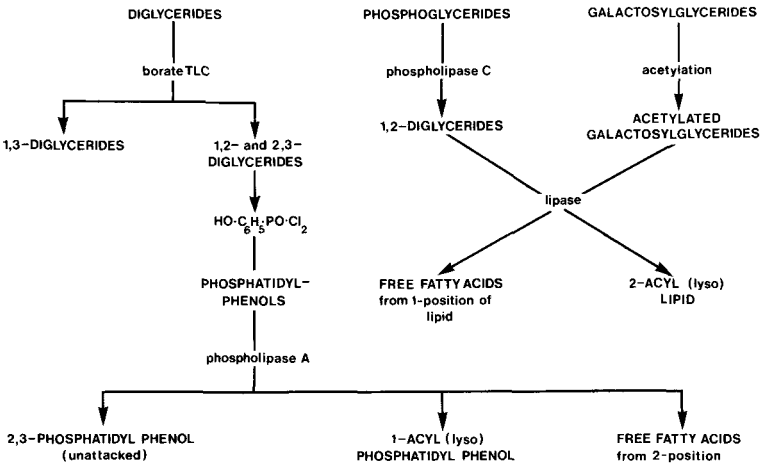

FIG. 2. Scheme for the analysis of diacyl glycerides.

Flour (100 g) was packed in a 20 x 3.5 cm dia. column and percolated with 1 liter diethyl ether. Ether was distilled off under nitrogen, and the residual lipid dissolved in chloroform for further analysis. This method was used for the rapid extraction of neutral lipids such as triglyceride (TG) and diglyceride (DG).

A complete extraction of flour lipids was achieved with water-saturated *n*-butanol, followed by purification and silicic acid column chromatography to give nonpolar lipid, glycolipid and phospholipid fractions (1). Individual lipid classes were then obtained by preparative TLC (2).

The distribution of fatty acids between the 1, 2, and 3 positions of the mono- and diacylglycerides was determined according to the schemes in Figures 1 and 2. To confirm the validity of the stereospecific analysis procedure, the composition of the fatty acids in the 1 and 2 positions of DG prepared from egg yolk phosphatidyl choline (PC) was determined and was found to be in excellent agreement with results obtained by phospholipase A_2 analysis of the original egg yolk PC.

Choline phosphoglycerides were hydrolyzed with phospholipase C from *Clostridium welchii* (8) (Sigma Chemical Co., London) and ethanolamine phosphoglycerides were hydrolyzed with phospholipase C prepared from *Bacillus cereus* (9) (NCIB 6349, Torry Research Station, Aberdeen). PC was hydrolyzed with phospholipase A_2 (*Ophiophagus hannah* snake venom, Sigma Chemical Co., London) in ethereal solution (10), and the other phosphoglycerides were hydrolyzed in collidene buffer (11) at pH 7.5. The products, free fatty acids (FFA) and 1-acyl lysophosphoglycerides, were separated by preparative TLC for further analysis (10).

Esterified (6-O-acyl) monogalactosyl monoglyceride (EMGalDG) was partially hydrolyzed in 2% (w/v) aqueous sulfuric acid for 2 hr at 100 C to give a mixture of FFA, DG, monoglycerides (MG) and acylated galactose. DG isolated by preparative TLC was assumed to contain the same fatty acids as the DG moiety of EMGalDG. The composition of the 6-O-acyl residue was calculated by difference.

Galactosyl diglycerides as such were unsuitable for quantitative argentation TLC or for pancreatic lipolysis. Monogalactosyl diglyceride (MGalDG) and digalactosyl diglyceride (DGalDG) were therefore acetylated with acetic anhydride in pyridine (12). The acetylated lipids were found to behave like DG on TLC and were readily hydrolyzed with pancreatic lipase. It is presumed that acetylated galactosyl diglycerides are specifically hydrolyzed at the 1 position by pancreatic lipase, as are galactosyl diglycerides (13,14), in accordance with the established stereospecificity of the enzyme (15).

TG, DG and acetylated MGalDG and DGalDG were hydrolyzed with ether-defatted porcine pancreatic lipase (16) (Koch-Light Labs., Colnbrook) at pH 8 and the products separated by preparative TLC. TG, DG, MG and FFA were separated by preparative TLC on Silica Gel G (E. Merck, Darmstadt, West Germany) using diethyl ether-benzene-ethanol-acetic acid (40:50:2:0.2 v/v), or chloroform-methanol-33% (w/v) ammonia-water (65:30:5:2.5 v/v) (2). Isomeric MG and DG were separated by TLC on Silica Gel G plates impregnated with 4% sodium tetraborate (17). Isomerization of DG was prevented by using borate-impregnated TLC plates (18) and by avoiding contact with water or methanol at

TABLE I

Fatty Acid Composition (Mole %) of Free Fatty Acids and Principal Glycerolipids in High Grade Winter Wheat Flour

FA	FFA	MG	DG	TG	MGalDG	DGalDG	EMGalDG	EMGalDG[a]	PE	PC	LPE	LPC
16:0	18.6	17.6	17.2	16.0	5.4	14.0	23.4	12.2	16.9	20.1	34.8	40.7
16:1	0.9	0.6	0.8	0.8	0.2	0.2	0.7	0.7	0.5	0.1	0.3	0.4
18:0	1.4	1.3	1.1	1.6	0.5	1.0	1.3	1.5	1.5	1.5	1.0	1.3
18:1	9.9	12.4	12.7	13.8	7.3	5.8	5.9	7.3	10.0	12.0	5.7	6.0
18:2	64.9	64.8	63.9	63.1	82.1	73.5	63.4	73.2	67.2	63.3	54.8	49.0
18:3	4.3	3.3	4.3	4.7	4.5	5.5	5.3	5.1	3.9	3.0	3.4	2.6

[a]DG core of EMGalDG only (see text).

extreme pH values during handling. Lipid bands were detected by spraying with ethanolic 2'7'-dichlorofluorescein and viewing under ultraviolet light. Lipids were extracted with diethyl ether (DG) or with monophasic chloroform-methanol-water (19).

Stereospecific analysis of TG and DG via phosphatidyl phenol derivatives (16,20) was performed on 5-10 mg samples using the above phospholipase A_2 and pancreatic lipolysis procedures. Fatty acid methyl esters were prepared from lipids with boron fluoride-methanol (21), and were analyzed by gas liquid chromatography (GLC) on polar (EGSS-X) columns (13).

DG, DG from PC or phosphatidyl ethanolamine (PE), acetylated MGalDG, or acetylated DGalDG were separated into fractions containing 0-6 double bonds per molecule by preparative TLC on Silica Gel G plates impregnated with 5% silver nitrate, developed in chloroform-ethanol (98.5:1.5 v/v). Bands were detected with 2'7'-dichlorofluorescein, recovered as described above, and subjected to pancreatic lipolysis and fatty acid analysis. Lipids were also applied as spots and the developed plates charred with 50% (v/v) sulfuric acid. The plates were then measured by densitometry using a Joyce-Loebl Chromoscan densitometer.

The validity of the densitometric quantitation was checked by adding a 17:0 fatty acid internal standard to each band and analyzing the fatty acid methyl esters from each band by GLC. Typical results with DG from wheat PC measured by densitometry and by GLC were, respectively: monoenes, 6.2 ± 0.5% and 7.0 ± 0.4%; dienes, 32.1 ± 0.4% and 31.0 ± 0.3%; and tetraenes, 40.6 ± 0.3% and 40.7 ± 0.8%. Figures given in the Results are the averages of three to six analyses and were not statistically evaluated. Fatty acid compositions are averages of three analyses with an error of about ±5% of the values given for major components and ±10% for minor components.

RESULTS AND DISCUSSION

The fatty acid compositions of the principal glycerolipids (Table I) in the sample of high grade winter wheat flour are very similar to those in high grade spring wheat flour (1), given in weight per cent. Linoleic acid is the principal fatty acid in each lipid and is most abundant in MGalDG. The lysophosphoglycerides lysophosphatidyl ethanolamine (LPE) and lysophosphatidyl choline (LPC) are the most saturated of the glycerolipids.

The stereospecific distribution of the fatty acids in TG and DG is given in Table II and

TABLE II

Stereospecific Distribution of Fatty Acids (Mole %)
in High Grade Winter Wheat Flour Diglycerides and Triglycerides

Lipids	Fatty Acid	Fatty acid in Fig. 3	Fatty acid 16:0	16:1	18:0	18:1	18:2	18:3
1,2-DG	Total	A + B	19.7	0.2	1.3	10.7	63.8	4.2
	1 Position	A	38.7	0.6	2.7	9.8	44.9	3.4
	2 Position	B[a]	0.9	-0.2	-0.1	11.6	82.7	5.0
2,3-DG	Total	A + B	17.5	0.3	1.2	11.8	64.6	4.5
	2 Position	A	37.1	0.6	2.4	9.8	46.1	4.0
	3 Position	B[a]	-2.1	-	-0.1	14.2	83.1	5.0
1,3-DG	Total	A + B	17.5	0.2	1.2	11.6	65.1	4.6
	1 Position	A	36.7	0.4	2.4	9.0	47.1	4.4
	3 Position	B[a]	-2.1	-	-0.1	14.2	83.1	5.0
1,2-(2,3)-DG	Total		18.1	0.4	1.5	12.2	63.6	4.2
	1 (3) Position		18.2	0.3	1.3	12.0	64.0	4.2
	2 Position		18.0	0.6	1.7	12.5	63.1	4.1
TG	Total		16.0	0.8	1.6	13.8	63.1	4.7
	1 Position		23.9	1.0	2.3	14.2	53.9	4.7
	2 Position		3.6	0.3	0.4	15.0	75.7	5.0
	3 Position		20.6	1.0	2.0	12.2	59.7	4.7

[a]Values determined by indirect calculation so that cumulative experimental error occasionally leads to small negative values.

Figure 3. The fatty acids in TG show the usual pattern of greater unsaturation at the 2 position (22). The 1 and 3 position fatty acids are very similar and wheat TG therefore resembles corn oil TG (16,20).

If DG are formed by the action of lipase on TG, isomeric DG of the type shown in Figure 4 would be expected, and the fatty acid compositions in all positions of the isomeric DG should be similar since 1 and 3 position fatty acids of TG are almost identical. This was not the case (Table II) and these results can only be explained if the DG isomers originated from 1,2-DG (Fig. 3). Since it is highly improbable that TG are selectively hydrolyzed at the 3 position, we conclude that the DG are either formed by breakdown of polar diacyl lipids, e.g., by β-galactosidase or phospholipase C, or that they are synthesized as such in the wheat grain. The pattern of lipid degradation in stored flours shows no evidence of galactosidase or phospholipase C types of activity (T.A. Clayton and W.R. Morrison, unpublished) and the latter conclusion is therefore favored.

If DG are the end products of biosynthesis, it is possible that the other partially-acylated glycerides are formed in the same way rather than by lipolysis. It is therefore interesting to calculate the FFA which would be released if all the partial glycerides were formed by lipolysis. Calculations (T.A. Clayton and W.R. Morrison, unpublished) show that the actual FFA levels are only about 40% of the calculated levels in three freshly-milled flours. Since FFA are not degraded in flour under normal storage conditions (T.A. Clayton and W.R. Morrison, unpublished) a substantial proportion of the partially acylated glycerides could not have been formed by lipolysis and must therefore have been synthesized as such.

The principal partially-acylated polar lipid in wheat flour is LPC (1). Acker and Schmitz (23) found that wheat starch LPC consisted of 78-80% 1-acyl isomer and 20-22% 2-acyl isomer. In this study LPE and LPC were both found to consist of about 80% 1-acyl isomer, but these figures may not be too significant because the lipids were in prolonged contact with silicic acid during their isolation and isomerization would reach equilibrium (24,25). Van den Bosch and Van Deenen (26) have stated that 2-acyl LPC will isomerize completely to 1-acyl LPC under certain conditions, but we have found that in our experimental procedures 1-acyl LPC, obtained by the action of phospholipase A_2 on egg yolk PC, will equilibrate to about 80% 1-acyl and 20% 2-acyl isomer.

The fatty acid compositions of the 1-acyl isomer of LPE and LPC (Table III) are very similar to those in the 1 positions of PE and PC respectively (Table IV). They could thus be formed by the action of phospholipase A_2 on the parent diacyl phosphoglycerides in a degradative reaction of the type found during flour storage (T.A. Clayton and W.R. Morrison,

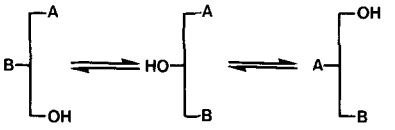

FIG. 3. Isomeric diglycerides after lipolysis of a triglyceride.

unpublished), or they could be synthesized as such. The fatty acids in the 2-acyl isomer of LPC are slightly more saturated than those of the 1-acyl isomer and are quite unlike those in the 2 position of PC. The 2-acyl isomer was probably an artifact produced by isomerization of 1-LPC. Amounts of LPE were very limited, and analyses of 2-acyl LPE were less satisfactory although they did exhibit the same pattern as 2-LPC.

Most of the other partially acylated glycerides were present in amounts too small for detailed analysis. Digalactosyl monoglyceride (DGalMG) and DGalDG have almost identical fatty acid compositions (1) which would suggest that the small amount of DGalMG present (1) is produced by random hydrolysis of DGalDG, cf., total composition (Table I) and 1 and 2 position fatty acid compositions (Table IV).

The MG isomers are very similar in fatty acid composition (Table III). Since there is little evidence for the monoglyceride pathway for TG synthesis in plants, they are unlikely biosynthetic intermediates. If MG are degradation products, their composition is consistent with their being formed by lipolysis of the isomeric DG (Fig. 3 and Table II) at the 1 and 3 positions so that the total fatty acid compositions of the DG and MG isomers are the same.

An explanation of these results is that some of the partial glycerides, e.g., DG, LPC, LPE, and FFA in flour were synthesized as such. They may have specific functions (why does wheat starch contain so much LPC?), or they may be biosynthetic intermediates; ripening grain contains significant amounts of DG (27-29) which is a key intermediate in the biosynthesis of most plant lipids (30). However there is no known biosynthetic route leading directly to 1-acyl LPC. Hydrolysis of PC to LPC would create large amounts of FFA which were not found. An alternative route might involve a PC: acceptor acyltransferase reaction of the type found in mammalian systems (31,32) or spinach leaf homogenates (33) but the nature of the esterified acceptor is not obvious. LPC is the principal lipid in wheat starch (23,34-37) and the nature of its distribution within intact starch granules (34,37) is hardly compatible with its being a degradation product of PC from other locations within the endosperm.

In addition to the lipids which are synthesized there are smaller amounts of partial glycerides, e.g., MG, DGalMG, and presumably some FFA which appear to be formed by random degradation at some time between the harvesting of the grain and the study of the milled flour. McKillican and Sims (38) claim that there are no FFA, MG or DG in ripe wheat, but Skarsaune et al. (27) and Pomeranz and Chung (29) found these lipids in wheat at all stages of grain maturation.

The fatty acid compositions of the di- and triacyl glycerides are given in Tables I, II and IV. PC may be synthesized from 1,2-DG and CDP-choline or from PE via the N-methylation pathway (30). Studies with ^{32}P-labeled substrates indicate that in anabolic plant tissue the former pathway predominates and in catabolic tissues the latter pathway predominates (39). The close similarity in fatty acid composition between the 1 and 2 positions of 1,2-DG and PC suggests direct synthesis from DG and CDP-choline. PE, which is a minor lipid during the early stages of grain maturation (27), has a slightly different fatty acid composition.

EMGalDG was originally isolated as an artifact from spinach leaf lipids (40) and is formed by enzymatic transfer of fatty acids from other galactosylglycerides or phosphoglycerides (33) onto the 6 position of galactose in MGalDG

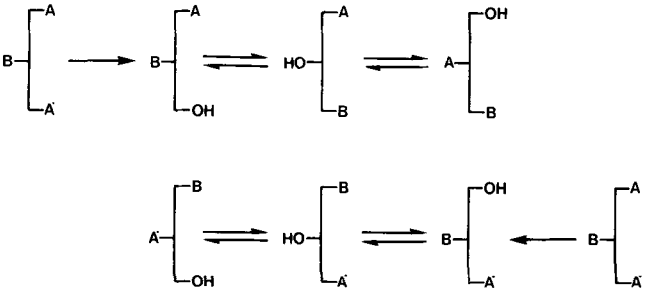

FIG. 4. Isomerization of *sn*-1,2-diacyl glycerol.

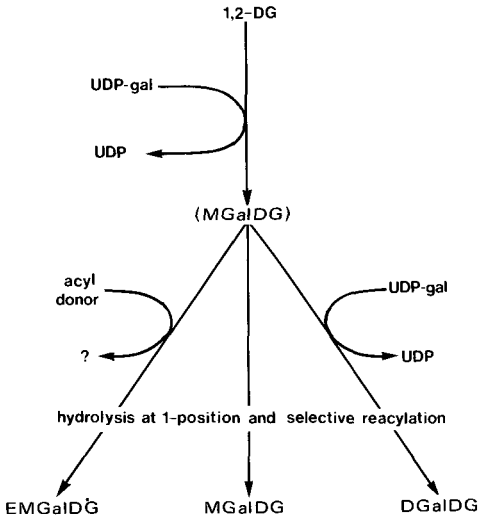

FIG. 5. Synthesis of galactosyl glycerides in wheat with suggested modification of 1 position fatty acids.

(41). The fatty acids in the DG moiety of EMGalDG are less unsaturated than those in MGalDG, and closely resemble those in DGalDG. The 6-O-acyl residue (Table III) is comparatively saturated and cannot arise by dismutation of the galactosyl diglycerides. The only sources of fatty acids of similar composition in wheat flour are the fatty acids in the 1 position of PC (Table IV) or LPC (Table III). These observations indicate that EMGalDG in wheat flour is probably not an artifact. The N-acyl fatty acids of N-acyl phosphatidyl ethanolamine are, in contrast, highly unsaturated (1) and, while not artifactual (1), are evidently incorporated by a different enzyme system.

The composition of fatty acids in the 1 and 2 positions of MGalDG and DGalDG are shown in Table IV. The 2 fatty acids are very similar to those in the 2 position of 1,2-DG, but the 1 fatty acids are not similar. If the galactosyl diglycerides are synthesized from 1,2-DG by established pathways (30) the selective placement of fatty acids could best be accounted for by hydrolysis of 1 fatty acids followed by re-esterification with acyltransferases of differing specificity for each lipid (Fig. 5). Alternatively selective utilization of DG or MGalDG molecular species, or both (42), may occur, although it seems less likely.

Lipids in maturing wheat are in a state of active synthesis, and there is turnover of the fatty acids resulting in changes in the fatty acid compositions of the principal lipid classes (27,28). Specific distributions of fatty acids of the type shown in Tables II and IV can be

TABLE III

Fatty Acid Composition (Mole %) of Monoacyl Lipids in High Grade Winter Wheat Flour

FA	FFA	LPE 1-isomer	LPC 1-isomer	LPC 2-isomer	MG 1 (3) isomer	MG 2-isomer	EMGalDG 6-O-acyl[a]	ESGlu[b]	SE[b]
16:0	18.6	34.5	40.6	44.8	17.4	17.5	45.8	56.3	62.6
16:1	0.9	0.3	0.5	0.7	0.9	0.7	0.7	1.6	
18:0	1.4	1.0	1.8	1.4	2.3	2.9		5.0	1.8
18:1	9.9	5.1	5.2	5.9	13.3	13.9	3.1	6.0	5.7
18:2	64.9	56.1	49.3	44.9	61.8	60.8	43.8	28.0	27.4
18:3	4.3	3.1	2.6	2.3	4.2	4.2	6.0	3.0	2.6

[a]Calculated from 3 x total fatty acid - 2 x DG moiety (Table I).

[b]From T.A. MacMurray and W.R. Morrison (1), results converted to mole %. ESGlu = esterified steryl glucoside, SE = steryl ester.

TABLE IV

Stereospecific Distribution of Fatty Acids (Mole %) in High Grade Winter Wheat Flour Phosphoglycerides and Galactosylglycerides

	Fatty acids in 1 position						Fatty acids in 2 position					
Lipid	16:0	16:1	18:0	18:1	18:2	18:3	16:0	16:1	18:0	18:1	18:2	18:3
PE	29.4	0.5	2.7	9.0	55.5	2.9	4.4	0.5	0.3	11.0	79.1	4.7
PC	37.8	---	2.7	7.8	49.2	2.5	2.4	0.2	0.3	16.2	77.4	3.5
MGalDG	10.8	0.3	0.9	5.4	81.2	1.4	0.2	0.1	0.1	9.2	83.0	7.4
DGalDG	26.3	0.3	1.7	4.3	63.4	4.2	1.9	0.2	0.3	7.3	83.0	7.3

attained by selective utilization of molecular species or by partial deacylation followed by selective reacylation, or both. There appears to be no proof of either mechanism in plant tissues in vivo (30).

Wheat flour lipids are derived from several anatomical parts of the grain and include substantial amounts of neutral lipids from the germ and scutellum (38,43,44). It is probable however that if selective utilization of molecular species had occurred there would still be a tendency for glyceride compositions to correspond to nonrandom fatty acid distributions, particularly in the polar lipids which are largely confined to endosperm.

The principal glycerolipids were separated by argentation TLC into classes containing 0-6 double bonds, and the fatty acids in the 1 and 2 positions of each fraction were determined. The principal glyceride molecular species were then calculated (Table V) and compared with values calculated for 1 random, 2 random fatty acid distributions. Triglycerides could not be resolved into enantiomorphic molecular species (45), but calculation on a 1 random, 2 random, 3 random fatty acid distribution basis gave stereospecific distributions of fatty acids in monoene, diene, and other triglyceride fractions which were close to experimental values. The glyceride distributions in several other triglycerides also correspond to 1 random, 2 random, 3 random fatty acid distributions (46,47), although there are notable exceptions.

The TG were also hydrolyzed with pancreatic lipase, the 1,2-DG + 2,3-DG isolated by preparative TLC and the molecular species of 1,2-DG determined by argentation TLC and stereospecific analyses of 1 and 2 position fatty acids (Table V). The experimental error in the results is difficult to assess, but in all the lipids studied the distributions of the principal molecular species appear to correspond to 1 random, 2 random fatty acid distributions.

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TABLE V

Principal Molecular Species (Mole %) of Wheat Flour Glycerolipids

Lipid fraction	Fatty acid		1,2-DG		1,2-DG from TG		PE		PC		MGalDG		DGalDG	
	1 position	2 position	Found	Calc.[a]	Found	Calc.[a]	Found	Calc.[a]	Found	Calc.[a]	Found	Calc.[a]	Found	Calc.[a]
Saturates	16:0	16:0	3.0	3.3		0.9		1.3		1.0				0.4
	16:0	18:0				0.1		0.1		0.1				0.1
	18:0	16:0				0.1	0.1	0.1		0.1				
Monoenes	16:0	16:1				0.1		0.1		0.1				
	16:0	18:1	5.4	4.5	3.5	3.6	4.5	3.2	6.1	6.1		1.0	2.1	1.9
	18:0	18:1	0.3	0.3	0.3	0.3	0.1	0.3	0.2	0.4		0.1	0.1	0.1
	18:1	16:0			0.5	0.5	0.5	0.4	0.2	0.2			0.1	0.1
Dienes	16:0	18:2	23.3	31.8	18.6	18.1	22.9	23.3	27.8	29.3	8.1	9.0	24.0	22.0
	18:0	18:2	1.8	2.2	1.7	1.7	2.2	2.1	2.0	2.1	0.6	0.7	1.5	1.4
	16:1	18:1	0.1	0.1		0.2		0.1	0.1					
	18:1	18:1	1.5	1.1	2.2	2.1	1.1	1.0	1.2	1.3	0.5	0.5	0.4	0.3
	18:2	16:0		0.4	2.0	1.9	2.4	2.4	1.0	1.1	0.2	0.2	1.1	1.1
	18:2	18:0			0.2	0.2		0.2	0.1	0.1	0.1	0.1	0.2	0.2
Trienes	16:0	18:3	1.9	1.9	1.1	1.2	1.4	1.4	1.3	1.3	0.4	0.8	2.3	1.8
	18:0	18:3	0.1	0.1		0.1		0.1	0.1	0.1		0.1	0.1	0.1
	16:1	18:2	0.6	0.5	0.7	0.8	0.4	0.4				0.2	0.4	0.3
	18:1	18:2	8.0	8.1	10.4	10.7	7.3	7.1	6.2	6.0	4.1	4.5	4.5	3.6
	18:2	16:1			0.1	0.2	0.3	0.3		0.1		0.1	0.1	0.1
	18:2	18:1	8.4	5.2	7.4	8.1	6.3	6.1	8.2	8.0	7.3	7.5	5.9	4.6
	18:3	16:0			0.2	0.2		0.1		0.1			0.1	0.1
Tetraenes	18:1	18:3	0.4	0.5	0.7	0.7	0.4	0.4	0.1	0.3	0.4	0.4	0.1	0.3
	18:2	18:2	39.6	37.1	41.6	40.8	44.3	43.9	39.8	38.1	70.1	67.4	48.6	53.0
	18:3	18:1	0.5	0.4	0.7	0.7	0.4	0.3	0.3	0.4	0.1	0.1	0.1	0.3
Pentaenes	18:2	18:3	2.6	2.2	2.7	2.7		2.6		1.7	5.5	6.0	4.2	4.4
	18:3	18:2	2.8	2.8	3.6	3.6		2.3		1.9	0.9	1.2	3.4	3.5
Hexaenes	18:3	18:3	0.2	0.2		0.2		0.1		0.1		0.1		0.3

[a]Calculated from data in Tables II and IV assuming 1 random, 2 random fatty acid distributions.

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Fatty Acid Specificity of Glyceride Synthesis by Homogenates of Bovine Mammary Tissue[1]

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ABSTRACT

Fatty acid esterification by cell free preparations of bovine mammary tissue was investigated to determine if the type of long chain fatty acid supplied might influence the rate of triglyceride synthesis by that tissue. Homogenates of lactating bovine mammary tissue esterified ^{14}C-fatty acids into glycerides at rates dependent upon chain length and degree of unsaturation. Palmitic, stearic, oleic and linoleic acids were esterified at rates consistent with their concentration in milk fat. A comparison of free fatty acid concentrations of mammary tissue with levels saturating esterification suggested that supply of fatty acids does not limit glyceride synthesis. Certain combinations of fatty acids were facilitory, competitive or inhibitory to esterification. Stearic acid complimented esterification of palmitic and oleic acids. Unlabeled *trans*-11-octadecenoic acid did not compete with ^{14}C-palmitate as efficiently in the esterification process as did unlabeled *cis*-9-octadecenoic acid, indicating that the mammary gland may preferentially esterify the *cis*-isomer of C-18:1. Linoleic acid inhibited esterification of palmitic, stearic and oleic acids.

The results of numerous in vitro studies, primarily with liver and intestinal mucosa, suggest that fatty acids are utilized at different rates for glyceride synthesis depending upon the number of carbon atoms and number, position and sterioisomerism of double bonds in their carbon skeleton (1-7). Specificity in glycerolipid synthesis may be imparted at several enzymic steps: acyl CoA synthetase (EC 6.2.1.3) (1,8,9), acyl transferase (EC 2.3.1.15) (2-4,7,10) or phosphatidate phosphohydrolase (EC 3.1.3.4) (11,12).

The influence of fatty acid on mammalian lipid biosynthetic reactions suggests that lipid metabolism may be regulated by diet. Responsiveness of milk fat synthesis to diet (14) together with the formation of a lipid product containing greater than 98% triglyceride commends mammary tissue for investigating the regulation of glyceride synthesis. Synthesis of glycerides by the mammary gland has been investigated in a variety of species (12,15-22), but with little regard to specificity of fatty acid esterification. The nonrandom distribution of fatty acids in bovine milk fat triglycerides suggests an ordered biosynthesis (23). Evidence is presented that long chain fatty acids are esterified by homogenates of bovine mammary tissue at rates dependent upon chain length, degree of unsaturation, and position of the double bond. These results support the concept (24) that triglyceride synthesis may be in part controlled by the type of long chain fatty acid supplied to the mammary gland.

A preliminary report of part of this work has been published (25).

METHODS

Preparation of Tissue

Bovine mammary tissue was prepared as previously described (26). Crude homogenates were centrifuged 800 x g for 10 min and filtered through glass wool. The resulting fil-

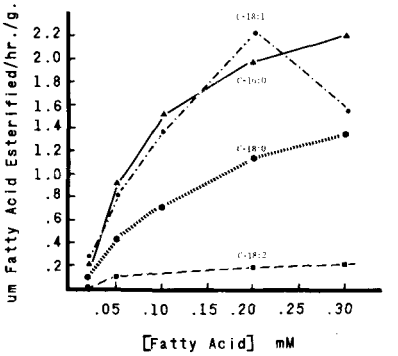

FIG. 1. Esterfication rates of several long chain fatty acids by bovine mammary homogenates. Conditions of assay were as described in Methods and Materials with 10.5 mM ATP plus albumin and DTT except each fatty acid substrate was varied as indicated and tested individually. Similar results were obtained under slightly different incubation conditions, i.e., substrate concentrations, in eight studies involving three animals.

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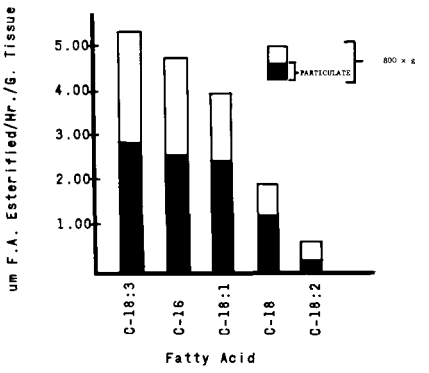

FIG. 2. Esterification of several long chain fatty acids by the 800 x g supernatant and particulate fraction of bovine mammary tissue. The esterification rate reported is the maximum value achieved over five substrate concentrations assayed (0 to 0.3 mM). Assay conditions are so described in Methods and Materials with 10.5 mM ATP plus albumin and DTT except substrate and enzyme source were varied as indicated. The 800 x g particulate fraction were derived from the same homogenate.

trate is referred to as homogenate. In some instances the homogenate was centrifuged 100,000 x g for 60 min and the particulate sediment resuspended in a volume of buffer equal to the homogenate from which it was derived.

Incubation Mixture

The sodium salts of 1-^{14}C fatty acids were diluted with their unlabeled analog to a specific activity of approximately 50,000 dpm/μmole fatty acid (26). The incubation mixture was optimized for palmitate esterification as described previously (26) and contained ATP (7.0 mM), CoA (0.4 mM), D,L-glycerol-3-phosphoric acid (20.0 mM), $MgCl_2$ (2.0 mM), NaF (50.0 mM), variable amounts of ^{14}C-fatty acid, 0.2 ml of a 1:8 homogenate containing approximately 2.0 mg protein, and phosphate buffer (90.0 mM) in a 2.0 ml incubation volume at pH 7.2. In some incubations bovine serum albumin (5 mg) and dithiothreitol (4.0 mM) were included in the incubation mixture. Their presence caused ATP requirements to increase to 10.5 mM and also increased the quantity of fatty acid required to saturate the enzyme. Incubations were at 37 C for 60 min with shaking. The reaction was terminated and product extracted by the addition of heptane-isopropanol-alkaline water (26).

Determination of Radioactivity and Expression of Results

A 2.0 ml aliquot of the heptane extracted lipids (Less than 4% of the ^{14}C-palmitate label extracted appeared in phospholipid and monoglycerides. Less than 2% of the label was FFA. The remainder was esterified in di and triglycerides.) was transferred to a glass scintillation vial. Scintillation fluid [Paradioxane (770 ml), xylene (770 ml), absolute ethanol (460 ml), 2,5-diphenyl-oxazole (10 g), α-naphthalphenyl-oxazole (100 mg), naphthalene (160 g).] was added and samples counted twice. Enzyme blanks were subtracted from each sample prior to calculation of results. Counting efficiency for internal standards was approximately 65% (26). Enzyme activity is expressed as μmoles of fatty acid esterified per hour per gram of tissue or per milligram extractable protein. Protein was determined by the method of Lowry et al. (27).

Chemicals

Chemicals used in this study and their sources are: Adenosene tri-phosphate, disodium salt (ATP) Coenzyme A, free acid (CoA), D,L-glycerol-3-phosphoric acid, disodium salt, bovine serum albumin, fraction V, and unlabeled fatty acids (Sigma Chemical Co., St. Louis, Missouri); dithiothreitol (Nutritional Biochemicals Corp., Cleveland, Ohio); 1-^{14}C-palmitic acid, 56.2 mC/mmole, 1-^{14}C stearic acid, 48.4 mC/mmole, 1-^{14}C-oleic acid, 43.2 mC/mmole, 1-^{14}C linoleic acid, 59.2 mC/mmole and 1-^{14}C linolenic acid, 41.5 mC/mmole (Amersham/Searle, Des Plaines, Illinois). Linoleic acid was also obtained from Hormel (Hormel Institute, Austin, Minnesota) and Applied Sciences (The Anspec Co., Ann Arbor, Michigan). Fatty acids were >98% pure by thin layer chromatography. All solvents used were reagent grade or higher.

RESULTS AND DISCUSSION

Esterification Rates of Individual Fatty Acids

Palmitic (C-16:0, hexadecanoic), stearic (C-18:0, octadecanoic), oleic (C-18:1, *cis*-9-octadecenoic) and linoleic (C-18:2, *cis, cis*-9-, 12-octadecadienoic) acids were tested for their ability to be esterified into glycerides by the 800 x g supernatant of mammary tissue from the lactating bovine. Typical esterification rates are shown in Figure 1. Oleic acid sometimes inhibited esterification at high concentrations. A similar inhibition by high concentrations of oleate has been noted for intestinal mucosa preparations (1,6). Linoleic acid was not esterified at rates comparable to the other acids. Esterification rates of palmitate and oleate were always similar. Incubations conducted in the absence of albumin and dithiothreitol and lower ATP levels (7.0 mM) required less fatty acid to achieve apparent maximum esterifica-

TABLE I

Competition Between Fatty Acids During Glyceride Synthesis[a]

Labeled acid	mM	Unlabeled acid	mM	μMoles ^{14}C-fatty acid esterified/hr/g	Per cent control
^{14}C-Palmitate	0.10	None		2.33	100
^{14}C-Stearate	0.10	None		0.66	100
^{14}C-Oleate	0.10	None		2.60	100
^{14}C-Palmitate	0.10	Palmitate	0.10	1.48	64
^{14}C-Palmitate	0.10	Stearate	0.10	2.19	94
^{14}C-Palmitate	0.10	Oleate	0.10	1.40	54
^{14}C-Stearate	0.10	Stearate	0.10	0.32	49
^{14}C-Stearate	0.10	Palmitate	0.10	0.21	32
^{14}C-Stearate	0.10	Oleate	0.10	0.30	46
^{14}C-Oleate	0.10	Oleate	0.10	1.40	54
^{14}C-Oleate	0.10	Palmitate	0.10	1.48	57
^{14}C-Oleate	0.10	Stearate	0.10	2.36	91

[a]These results are from a total of 12 incubations using the same enzyme source. Conditions of assay were as described in Methods with 7.0 mM ATP without albumin and DTT except fatty acid substrate was varied as indicated. Rate of fatty acid esterification refers to only the ^{14}C-fatty acid not total fatty acid. The esterification rate of each ^{14}C-fatty acid when incubated alone is referred to as 100%. The fatty acid concentration of 0.1 mM was in the range of substrate saturation of enzyme under these conditions.

tion rates. Regardless of the cofactors employed, the relative order of esterification indicated in Figure 1 was observed.

Unsaturated fatty acids in the 100,000 x g supernatant of various tissue preparations have stimulated glyceride synthesis (11). Fatty acid esterification was tested in the presence and absence of the 100,000 x g supernatant to determine if endogenous acids in the supernatant of mammary tissue homogenates would influence the fatty acid specificity of the particulate fraction. The same relative order of esterification of C-18:3, C-16:0, C-18:0, C-18:1 and C-18:2 was observed in the particulate fraction as in the 800 x g supernatant (Fig. 2). The absence of soluble phosphatidate phosphohydrolase or soluble acyl transferases (28) may also have contributed to the lower velocities of esterification observed in the particulate fraction. Unusual kinetics were displayed by C-18:3. At low to intermediate substrate concentrations the esterification rate of linolenic acid was less than that of palmitic or oleic acids, but at high substrate concentrations the esterification rate of linolenic acid exceeded that of either palmitic or oleic acid.

Tissue Concentration of Free Fatty Acids (FFA)

The 800 x g supernatant from mammary tissue of five cows contained 4.2 ± 0.5 μequiv. of FFA/g tissue. All the FFA present in the whole cell remained in the 800 x g supernatant and 84% of that FFA was associated with the particles sedimented at 100,000 x g. Since the enzymes of glyceride synthesis are also particulate (29) the local concentration of FFA may be much higher than 4.2 mM. This value is several times greater than the apparent range of substrate saturation determined for certain representative fatty acids and indicates that the supply of fatty acid may not limit glyceride synthesis in mammary tissue.

Free Acid Combinations

Stimulation of mammary gland palmitate esterification by various unlabeled FFA was investigated because such stimulations have been demonstrated in cat intestinal mucosa and to a limited extent in rat liver preparations (11,30). The esterification of 0.10 mM ^{14}C-palmitate was measured in the presence of unlabeled butyrate, palmitate, oleate, stearate and linoleate ranging from 0-0.10 mM. Unlabeled palmitate and oleate each decreased ^{14}C-palmitate esterification similarly but less than linoleate. Stearate and butyrate had little effect. Incubations of 0.10 mM ^{14}C-palmitate with combinations of 0.02 mM unlabeled stearic, oleic, linoleic and butyric acids were made to test the possibility that a combination of fatty acids might permit synthesis of a product with a balanced composition and thus stimulate palmitate esterification. No combination of 2-4

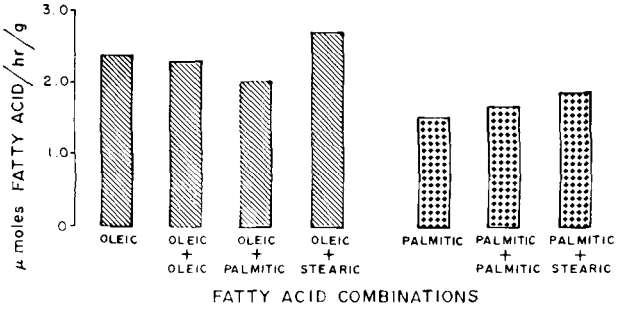

FIG. 3. Comparison of total fatty acid esterification of several combinations of equal specific activity fatty acids. ^{14}C oleic and ^{14}C palmitic were incubated separately at 0.10 mM concentrations and then with 0.10 mM ^{14}C palmitic, ^{14}C oleic or ^{14}C stearic acid. Combinations of fatty acids contained 0.20 mM total fatty acid. Conditions of assay were described in Methods with 7.0 mM ATP without albumin and DTT except fatty acid was varied as indicated.

unlabeled acids at 0.02 mM stimulated esterification of ^{14}C-palmitate. This was true regardless of whether the 800 x g or particulate fraction was used as an enzyme source.

Further studies were conducted with ^{14}C-palmitate, ^{14}C-oleate and ^{14}C-stearate tested separately and with each of the other unlabeled acids. Each labeled fatty acid, when incubated with its unlabeled analog, caused approximately a 50% decrease in incorporation of label as would be predicted by dilution of substrate specific activity (Table I). No combination of fatty acids increased esterification of any fatty acid above that noted when the fatty acid was incubated alone. Stearate did not decrease ^{14}C-palmitate or ^{14}C-oleate esterification appreciably, but both palmitate and oleate markedly decreased ^{14}C-stearate esterification. Oleate and palmitate each decreased the other acids' incorporation to a similar extent (54 and 57%), indicating that these two fatty acids competed similarly in the esterification process.

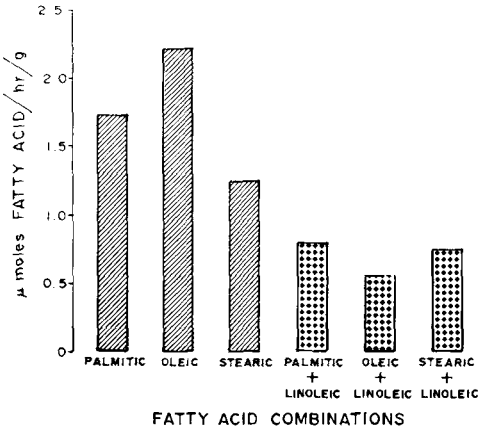

FIG. 4. Linoleate inhibition of fatty acid esterification. Each value represents the total fatty acid esterification in the presence of 0.20 mM ^{14}C fatty acid. All fatty acids were of equal specific activity. Combinations of fatty acids contained 0.10 mM of each fatty acid. Conditions of assay were as described in Methods with 7.0 mM ATP without albumin and DTT except fatty acid was varied as indicated.

Although combinations of fatty acids did not stimulate each other's esterification, the possibility existed that certain acids might be somewhat additive in their combined esterifications. Each ^{14}C-fatty acid of equal specific activity was incubated singly at 0.10 and 0.20 mM concentrations and then at 0.10 mM in various combinations with other 0.10 mM fatty acids. The results are shown in Figures 3 and 4. Since all fatty acids were ^{14}C labeled, only total fatty acids esterified and not the contribution of each acid to this total could be calculated. Stearate when incubated with palmitate or oleate resulted in a greater esterification of fatty acids than any of the other combinations (Fig. 3). Although the combination of palmitate and oleate resulted in a greater esterification than palmitate alone, the combined esterification was less than that of oleate alone.

The inhibitory effect of ^{14}C-linoleic acid on the esterification of three other ^{14}C-fatty acids is illustrated in Figure 4. This confirmed the inhibition indicated when unlabeled linoleate was included in the incubation mixture. Esterification of ^{14}C-fatty acid decreased approximately 50% each time 0.10 mM ^{14}C-linoleate was included in the incubation mixture. Inhibitory actions of such compounds may indicate nonspecific chemical or physical effects in an assay system rather than a specific cellular action. Detergent inhibition of enzyme action is indicated by nonlinear 1/V vs. "inhibitor" plots and a dependency upon protein to detergent ratios in the incubation mixture (5,31-33). Tests of these criteria with regard to linoleate

inhibition of fatty acid esterification by mammary homogenates failed to indicate detergent action by linoleate (34). Furthermore inhibition of fatty acid esterification was unique to C-18:2; neither C-16:0, C-18:0, C-18:1, nor C-18:3 were inhibitory. Three different sources of linoleate in studies involving tissue from nine animals were all inhibitory. The concentrations of linoleate found to be inhibitory were similar to endogenous concentrations of linoleate found in mammary tissue (0.07 μmoles/g, 2 animals). Thus linoleate inhibition of fatty acid esterification may be of biological importance in fatty acid esterification by the bovine mammary gland. Linoleate has been reported to inhibit stearate desaturase activity of goat mammary tissue (35) and fatty acid synthesis by mouse liver (13,36), indicating that this fatty acid may function as a regulator of fatty acid metabolism.

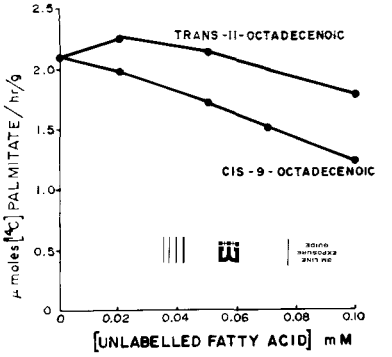

FIG. 5. ^{14}C palmitate esterification in the presence of unlabeled *cis* or *trans* isomers of octadecenoic acid. Esterification of 0.10 mM ^{14}C palmitate was measured in the presence of increasing concentrations on either *trans*-11-octadecenoic or *cis*-9-octadecenoic acid. Conditions of assay were as described in Methods with 7.0 mM ATP without albumin and DTT except fatty acid was varied as indicated.

Effect of *Cis-Trans* Isomerism and Double Bond Position

Feeding restricted roughage high grain rations to dairy cattle has resulted in an increased production of a *trans*-isomer of C-18:1 due to altered ruminal hydrogenation of linoleic acid (37,38). Unlabeled *cis*-9-octadecenoic acid decreased ^{14}C-palmitate esterification to a greater extent than did *trans*-11-octadecenoic acid, indirectly indicating that the *cis*-isomer (oleic) of C-18:1 may have been esterified more readily than the *trans*-isomer (vaccenic) of C-18:1 (Fig. 5). However an inhibitory effect of *trans* C-18:1 was not ruled out since ^{14}C-labeled *trans* C-18:1 was not tested with ^{14}C-palmitate for its effects on total fatty acid esterification.

In vitro esterification rates were in general agreement with the composition of milk fat, i.e., C-16:0~C-18:1>C-18:0>C-18:2. The esterification rate of C-18:3 however far exceeded its concentration in milk fat, but ruminal hydrogenation precludes significant quantities of this acid from reaching the mammary gland (39). Mammary tissue FFA concentrations were sufficient to saturate the in vitro esterifying system. Stearate facilitated fatty acid esterification when combined with palmitate or oleate. Studies suggested that the *trans* isomer of C-18:1 was not esterified as well as the *cis* isomer. Linoleic acid was poorly esterified by mammary tissue and markedly inhibited the esterification of other fatty acids.

The fatty acid specificities observed in this and other studies (40) may be related to the known responsiveness of milk fat production to dietary alterations. McCarthy et al. (41) have proposed that mammary gland will efficiently utilize only those fatty acids which fit the normal pattern of milk fat composition. The results of this study agree with and extend this concept.

ACKNOWLEDGMENTS

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The Hypolipidemic Effect of SU-13,437 in Rats With Natural Endogenous Hypertriglyceridemia

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ABSTRACT

The oral administration of 25 mg 2-methyl-2-[p-(1,2,3,4-tetrahydro-1-naphthyl)-phenoxy] propionic acid (SU-13,437) per kilogram body weight per day in two-month-old female rats with naturally occurring hypertriglyceridemia resulted in a 31% decrease ($P<0.01$) in the serum triglyceride concentration after 14 days of treatment, compared with littermate controls receiving diluent (Polyethylene glycol-300) only. Phospholipid, cholesterol, free fatty acid, glucose, red blood cell and white blood cell concentrations were similar in the blood of treated and control animals after 14 days of treatment. Liver total and relative weight was increased as a result of SU-13,437 treatment ($P<0.01$). The liver total lipid concentration per 10 gm liver decreased ($P<0.05$) due to a decrease in triglyceride ($P<0.01$), cholesterol ($P<0.01$), and free fatty acid ($P<0.05$) concentration following treatment. The relative liver glycogen concentration was elevated in treated rats at 7 ($P<0.01$) and 14 ($P<0.05$) days of treatment.

INTRODUCTION

The prevalence of familial hyperlipoproteinemias (1-4) as well as the direct relationship between hyperlipidemia and atherosclerosis in the human population (4-7) has indicated a need for blood lipid lowering compounds. The compound 2-methyl-2-[p-(1,2,3,4-tetrahydro-1-naphthyl)-phenoxy] propionic acid (SU-13,437) is known to lower serum cholesterol and triglycerides in experimental animals and

TABLE I

Effect of SU-13,437 on the Body Weight and Blood Composition of Naturally Occurring Hypertriglyceridemic Female Rats[a] After 7 and 14 Days of Treatment

Treatment	SU-13,437 x 7 days[b]	PEG-300 x 7 days[c]	SU-13,437 x 14 days[b]	PEG-300 x 14 days[d]
Body weight, g, day 0	235 ± 6[e]	225 ± 4	225 ± 8	224 ± 8
Body weight, g, change	±20 ± 6	±18 ± 5	±13 ± 4	±16 ± 4
Blood				
Triglycerides, mg/100 ml serum	641 ± 42	770 ± 75	625 ± 26	912 ± 72
Phospholipids, mg/100 ml serum	143 ± 8	150 ± 5	124 ± 5	135 ± 8
Cholesterol, mg/100 ml serum	75 ± 3	70 ± 4	59 ± 4	54 ± 4
Free fatty acids, uEq/liter serum	312 ± 6	179 ± 9	174 ± 11	206 ±11
Glucose, mg/100 ml plasma	109 ± 3	105 ± 4	108 ± 6	95 ± 2
Red blood cells millions/mm^3 whole blood			2.97 ± 0.11	2.80 ± 0.09
White blood cells thousands/mm^3 whole blood			11.18 ± 0.41	10.95 ± 1.01

[a]There were 10 rats per group (representing 7 litters) and they were 71 and 80 days of age at autopsy in the 7 and 14 day studies, respectively.

[b]25 mg SU-13,437 dissolved in 1 ml Polyethylene glycol-300 (PEG-300); 1 ml/kg body weight administered daily by gavage at 7 A.M.

[c]Littermates to group receiving SU-13,437 x 7 days; PEG-300 only, 1 ml/kg body weight administered.

[d]Littermates to group receiving SU-14,437 x 14 days; PEG-300 only, 1 ml/kg body weight administered.

[e]Mean ± standard deviation.

TABLE II

Effect of SU-13,437 on Liver Weight and Composition of Naturally Occurring Hypertriglyceridemic Female Rats[a] After 7 and 14 Days of Treatment

Treatment	SU-13,437 x 7 days[b]	PEG-300 x 7 days[c]	SU-13,437 x 14 days[b]	PEG-300 x 14 days[d]
Body weight, g, at autopsy (day 7 or 14)	236 ± 6[e]	228 ± 4	219 ± 7	222 ± 1
Liver relative weight, g/100 g body weight	3.78 ± 0.05	3.07 ± 0.04	3.83 ± 0.06	2.71 ± 0.10
Liver relative composition (per g liver)				
Total lipid, mg	35.3 ± 1.0	44.3 ± 1.6	30.9 ± 1.8	37.9 ± 2.5
Triglyceride, mg	4.3 ± 0.3	12.2 ± 1.1	7.0 ± 0.5	17.4 ± 1.0
Phospholipid, mg	26.6 ± 0.9	26.7 ± 1.0	26.4 ± 0.8	26.0 ± 0.4
Cholesterol, mg	2.3 ± 0.1	3.1 ± 0.1	2.2 ± 0.1	3.0 ± 0.1
Free fatty acids, uEq	8.7 ± 0.7	12.8 ± 0.8	14.0 ± 1.5	29.3 ± 1.7
Glycogen, mg	2.1 ± 0.4	0.6 ± 0.1	2.5 ± 0.6	0.6 ± 0.2

[a]There were 10 rats per group (representing 7 litters) and they were 71 and 80 days of age at autopsy in the 7 and 14 day studies, respectively.

[b]25 mg SU-13,437 dissolved in 1 ml Polyethylene glycol-300 (PEG-300); 1 ml/kg body weight administered daily by gavage at 7 A.M.

[c]Littermates to group receiving SU-13,437 x 7 days; PEG-300 only, 1 ml/kg body weight administered.

[d]Littermates to group receiving SU-13,437 x 14 days; PEG-300 only, 1 ml/kg body weight administered.

[e]Mean ± standard deviation.

man (8-24). However the study of this compound in experimental animals having abnormal lipid patterns has been limited. The availability of an inbred rat colony in which all animals have naturally occurring hypertriglyceridemia apparently equivalent to Fredrickson phenotype IV (25) prompted the study reported here. Two-month-old female rats were chosen for this study since it has been shown previously that females of this strain have higher serum triglycerides than males (26) and the serum triglyceride concentration is significantly elevated by two months of age (25).

MATERIALS AND METHODS

Forty female rats representing seven litters from an inbred colony of rats with natural endogenous hypertriglyceridemia (Substrain of the Long-Evans strain) were weaned and separated from their male littermates at 22 days of age. At two months of age the females from each of four of the litters were divided equally into an experimental and control group of 10 rats each for a 7 day study and those from each of the remaining three litters were divided equally into an additional experimental and control group of 10 rats each for a 14 day study. All rats were weighed and gavaged daily at approximately 7:00 AM during the ensuing experimental periods. The experimental groups received 25 mg SU-13,437/kg body weight daily, dissolved in polyethylene glycol (PEG-300) (25 mg/ml), whereas the littermate control groups received 1 ml of PEG-300/kg body weight daily. All animals were provided with commercial laboratory chow (Purina Laboratory Chow) and water ad lib. On the last day of each experimental period, following an 18 hr fast, the rats from one experimental and one control group were anesthetized with 50 mg sodium pentobarbital per kilogram body weight. One heparinized hematocrit tube of tail vein blood was obtained from each rat, centrifuged, and the plasma immediately assayed for true glucose by an ultramicro modification of the glucose oxidase-peroxidase method (27). In the two week study red and white cell counts were performed on 20 μl of tail vein blood using the Unopette method adapted for the Fisher Autocytometer (28). The animals were then exsanguinated via the dorsal aorta by means of disposable syringes fitted with 20 gauge disposable needles. The blood was allowed to clot at room temperature and centrifuged. The serum was assayed for lipids without delay.

Immediately after removal a 1 g piece of liver was carefully weighed and digested in 4 ml of hot 30% KOH for glycogen determination by the method of Seifter (29). The remainder of the liver was weighed and extracted for lipid analysis as previously described (30).

Serum and liver extracts were analyzed for phospholipids (31), cholesterol (32), triglycerides (expressed as triolein) (32), and free fatty acids (34) by automated methods. Liver total lipids were determined gravimetrically.

Analyses for changes were performed on the

data by use of appropriate estimates of error and student t-tests (35).

RESULTS

Rats treated with SU-13,437 gained as much weight as their respective littermate controls after 7 and 14 days of treatment (Table I). The final body weights (for body weight change determinations only) were taken 24 hr prior to autopsy because all animals were fasted for a period of 18 hr before autopsy.

Blood lipids, glucose, and red and white cell concentrations in treated and control animals are given in Table I. Serum triglycerides were slightly lower in SU-13,437 treated animals than in littermate controls after seven days of treatment but were 31% lower ($P<0.01$) in treated than control animals after 14 days of treatment. Serum phospholipid and cholesterol concentrations were not affected by SU-13,437. While serum free fatty acid concentrations were 74% higher ($P<0.01$) in SU-13,437 treated animals than in littermate controls after seven days of treatment, there was no difference between treated and littermate control rats after 14 days of treatment. Plasma glucose concentrations were not altered as a result of SU-13.437 treatment. The red and white blood cell concentrations were determined only after 14 days of treatment and were found to be similar in treated and control rats.

The effect of SU-13,437 on relative liver weight, lipid and glycogen concentrations was evaluated (Table II). Liver relative weights were 23% and 41% higher in SU-13,437 treated animals than in littermate controls after 7 and 14 days of treatment, respectively ($P<0.01$), indicating that the compound produced hepatomegaly in this experimental animal. Liver total lipid concentrations were 20% ($P<0.01$) and 18% ($P<0.05$) lower in the SU-13,437 treated rats than in their littermate controls after 7 and 14 days of treatment, respectively. The lower liver total lipid concentrations were due mainly to the 64% and 60% lower liver triglyceride concentrations in the treated rats compared with their controls after 7 and 14 days of treatment, respectively ($P<0.01$). Other contributing factors to the lower total lipid concentrations were the 26% ($P<0.01$) and 27% ($P<0.01$) lower liver cholesterol and 32% ($P<0.01$) and 52% ($P<0.05$) lower liver free fatty acid concentrations in the treated rats compared to their littermate controls after 7 and 14 days of treatment, respectively. Liver phospholipid concentrations were similar in treated and control rats. Liver relative glycogen concentrations were 247% ($P<0.01$) and 300% ($P<0.05$) higher in SU-13,437 treated rats than in their littermate controls at 7 and 14 days of treatment, respectively.

DISCUSSION

There was a 31% lower serum triglyceride concentration in naturally occurring hypertriglyceridemic female rats treated orally with SU-13,437 for 14 days compared to littermate controls receiving vehicle only. The serum triglyceride response observed in this study was similar to that observed in previous studies with normal (8,9,10,13) and fructose-induced hypertriglyceridemic (8,10) rats, and in humans with the Fredrickson-Lees-Levy phenotypes II, III, IV and V (11,12). Serum cholesterol levels were not affected by SU-13,437 in this study, in agreement with previous studies in Charles River rats by Best and Duncan (9,13). On the contrary a serum cholesterol lowering effect by SU-13,437 has been observed in male albino rats (8). Human studies have demonstrated varying degrees of hypocholesterolemic effect (9,11,12). Since rats like humans appear to have phenotypes, it appears plausible that not all rat strains (phenotypes) will respond in the same fashion.

In human studies on patients with various lipoproteinemias, predominantly Fredrickson phenotype II, the white blood cell count was decreased after treatment with SU-13,437 (12). In the current studies a decrease in the white blood cell count was not observed.

Hepatomegaly was observed in the current studies and has been reported in previous studies employing SU-13,437 in the rat (8,9,10,13). Liver total lipid concentration decreased due mainly to a decrease in the triglyceride. The currently reported decrease in liver triglyceride and cholesterol is supported by previous studies in the rat (8,13).

Relative liver glycogen concentrations were three times greater in the treated animals than in their littermate controls. On the contrary Hess and Bencze (8) found that liver glycogen concentration was lower in male albino rats treated with two fifths the amount of SU-13,437 utilized in the current study. Liver glycogen like serum cholesterol may be affected differently in different rat strains.

Maragoudakis has shown that SU-13,437 is a competitive inhibitor of acetyl CoA carboxylase in vitro in chicken (36--38) and rat (39,40) liver. Competitive inhibition in vivo would limit or prevent the synthesis of fatty acids from acetyl CoA, depending on the ratio of inhibitor/substrate present. The decrease in liver fatty acids, triglycerides and total lipids as well as the decrease in serum triglycerides observed in the current studies could support the hy-

pothesis that free fatty acid synthesis is diminished by the drug, particularly since the hypertriglyceridemia shown by the strain of rats used here is carbohydrate induced. Since we are dealing here with animals which have been fasted for 18 hr, the lower hepatic free fatty acid concentration in the SU-13,437 treated animal may be due to a difference in the rate of fatty acid utilization. The greater liver glycogen concentration in treated animals may be the result of decreased conversion of dietary glucose to acetyl CoA, free fatty acids and triglyceride, leading to greater conversion of glucose to glycogen in this hypertriglyceridemic rat. More experimental work must be conducted to clarify the effect of SU-13,437 on glycogen synthesis and storage.

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The in Vivo Incorporation of Labeled Linoleic Acid, α-Linolenic Acid and Arachidonic Acid Into Rat Liver Lipids

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ABSTRACT

The incorporation of 1-^{14}C-linoleic acid, 1-^{14}C-α-linolenic acid and 1-^{14}C-arachidonic acid into rat liver lipids was measured and the per cent distribution of radioactivity into the different lipid fractions determined. Normal rats were injected into the portal vein with the labeled solutions during a one minute period. Livers were quickly frozen, pulverized, and the lipids extracted and fractioned by thin layer chromatography. No significant differences were observed in the amounts of labeled fatty acids incorporated per gram of rat liver. While 1-^{14}C-linoleic acid and 1-^{14}C-α-linolenic acid were found in appreciable amounts in the 1,2 diacylglycerol fraction, about one fifth as much 1-^{14}C-arachidonic acid was esterified in this fraction. 1-^{14}C-arachidonic acid was the leading acid esterified in the phospholipid fractions.

INTRODUCTION

It is well known that the fatty acids of the linoleic acid family play an important role in the structure of phospholipids of mammalian tissues.

The incorporation of these acids has been studied either with in vitro methods (1-3), in which the normal balance of cell metabolism was necessarily upset, or with in vivo methods (4-6), employing rats fed different diets during rather long periods of time. Hill et al. (3) reported a great difference in the incorporation and distribution of fatty acids into lipids when incubated solubilized preparations from pigeon liver microsomes were compared with the results obtained with incubation of liver slices. Elovson (7) demonstrated the early course of in vivo desaturation and esterification of 1-^{14}C-stearic acid in rat liver. More recently Akesson (8) reported the in vivo rate of incorporation of labeled linoleic acid into rat liver lipids.

In earlier experiments (9) we studied the simultaneous incorporation of 1-^{14}C-linoleic acid and ^{3}H arachidonic acid into liver lipids from normal and diabetic rats. In the hope of obtaining further insight into the metabolism of liver lipids in intact normal cells, the present study concerns itself with the relative in vivo incorporation and molecular distribution of 1-^{14}C-linoleic acid, 1-^{14}C-α-linolenic acid and 1-^{14}C-arachidonic acid.

MATERIAL AND METHODS

1-^{14}C-linoleic acid (52.9 mC/mmol), 1-^{14}C-α-linolenic acid (41.5 mC/mmol) were purchased from Radiochemical Centre, Amersham, England. They were both 98% radiochemically pure. 1-^{14}C-arachidonic acid (53.0 mC/mmol) was purchased from Applied Science, U.S., and was 99% radiochemically pure. 1-^{14}C-linoleic acid and 1-^{14}C-α-linolenic acid had 2% *cis-trans*-unsaturated acid, and 1-^{14}C-arachidonic had 65% all *cis*. Phospholipase A was obtained from Ross Allen Institute, U.S.

Animals

Male albino Wistar rats weighing 150-170 g and fed Purina chow were used. The animals were fasted during the 6 hr prior to the experiment.

Preparation of Injection Solution

Each fatty acid was dissolved in 0.05 ml of 0.1 N KOH and the solution was heated at 25 C until a nearly clear soap solution was obtained. Then 0.6 ml of rat serum was added and after standing overnight in N_2 atmosphere at room temperature with magnetic stirrer this solution

TABLE I

Amount of Labeled Fatty Acids Incorporated Into Rat Liver Lipids

Fatty acid	No. of animals	nMoles/g rat tissue[a]
C18:2	6	3.45 ± 1.25
		N.S.
αC18:3	7	6.17 ± 1.50
		N.S.
C20:4	5	5.07 ± 1.01

[a]The data are the means ± standard deviations of the mean. Probability (P) values are related to arachidonic acid (C20:4). See Methods.

[1]Member of the National Council of Technical and Scientific Research of Argentina.

TABLE II

Amount of Labeled Fatty Acids Incorporated Into Lipid Fractions From Rat Liver

Fatty acid	No. of animals	nMoles/g wet tissue[a]			
		1-2 Diacylglycerol	Triacylglycerol	3-*sn*-Phosphatidylcholine	3-*sn*-Phosphatidylethanolamine
C18:2	6	0.92 ± 0.13 P <0.001	1.32 ± 0.14 N.S.	0.63 ± 0.13 P <0.02	0.17 ± 0.04 P <0.001
αC18:3	7	1.06 ± 0.08 P <0.001	2.48 ± 0.23 N.S.	1.51 ± 0.35 N.S.	0.27 ± 0.07 P <0.001
C20:4	5	0.20 ± 0.03	2.01 ± 0.24	1.89 ± 0.23	0.48 ± 0.04

[a]Data are the means ± standard deviations of the means. Probability (P) values are related to arachidonic acid (C20:4).

was used directly, 0.1 ml contained 10 μc of labeled fatty acid.

Injection of Labeled Solution

Groups of five or more rats were anesthetized with ether and the portal vein was exposed. The labeled solution, 0.1 ml per rat, was injected at a fairly constant rate during a 1 min period. Immediately following injection the liver was frozen between two aluminum blocks that had been precooled in liquid N_2, pulverized in a precooled mortar and extracted by the method of Folch et al. (10).

The extract was concentrated and the lipids were separated by thin layer chromatography (TLC).

Separation of Lipids

Phospholipids were separated into their components from the original lipid extract by TLC in chloroform-methanol-water (65:25:4 v/v) (11).

The spots, identified by comparison with authentic standards and visualized by I_2 vapors, were scraped off, transesterified with methanolic HCl (12), extracted with light petroleum, evaporated and assayed for radioactivity.

Neutral lipids were also separated into their components by TLC in petroleum ether-ethyl ether-acetic acid (80:20:1 v/v) (13) and assayed as mentioned above. The over-all recovery of radioactivity after transesterification was 70-90%.

However, since in liver extracts cholesterol stains too deeply to permit direct observation of diglyceride, this whole region was routinely scraped off as one band, saponified with 5% KOH in methanol, cholesterol extracted with petroleum ether, the water soluble layer acidified, and the free fatty acids derived from diglycerides extracted with light petroleum, evaporated and assayed for radioactivity.

Phospholipid Hydrolysis

Phosphatidylcholine and phosphatidylethanolamine, separated by TLC as described, were extracted from the silica using the two phase system of Arvidson (14).

After evaporation of the solvent the phospholipids were dissolved in 5 ml of diethyl ether and to this was added 1 mg of Crotalus adamanteus venom in 0.2 ml of 1 M borate buffer pH 7.0, containing 0.04 M calcium acetate. The ether-buffer system was shaken vigor-

TABLE III

Positional Distribution of Radioactive Fatty Acids Incorporated Into 3-*sn*-Phosphatidylcholine and 3-*sn*-Phosphatidylethanolamine

Fatty acid	No. of animals	3-*sn*-Phosphatidylcholine[a]		3-*sn*-Phosphatidylethanolamine[a]	
		1 Position, %	2 Position, %	1 Position, %	2 Position, %
C18:2	6	5.2 ± 2.8 P <0.05	94.8 ± 2.8 P <0.05	12.6 ± 5.0 P <0.05	87.4 ± 5.0 P <0.05
αC18:3	7	32.5 ± 2.5 P <0.001	67.5 ± 2.5 P <0.001	35.7 ± 3.0 P <0.001	64.3 ± 3.0 P <0.001
C20:4	5	1.2 ± 0.5	98.8 ± 0.5	5.0 ± 2.5	95.0 ± 2.5

[a]Data are the means ± standard deviations of the means. Probability (P) values are related to arachidonic acid (C20:4).

ously at room temperature overnight. Following hydrolysis the mixture was dried under reduced pressure. The dried lipids were dissolved in chloroform-methanol (9:1 v/v) and the free fatty acids and lysoderivates were separated by TLC as described previously and assayed for radioactivity.

Radioactivity Measurements

Radioactivity measurements were performed in a Packard Tri-Carb Scintillation spectrometer, Model 3003, with a solution of 2.5 diphenyl oxazol (PPO 5g/liter) and 1.4-bis-4-methyl-5-phenyl-oxazolyl-(2) benzol (dimethyl-POPOP 0.3 g/liter) in toluene.

RESULTS

The capacity of the liver to esterify the injected 1-^{14}C-linoleic acid, 1-^{14}C-α-linolenic acid and 1-^{14}C-arachidonic acid is summarized in Table I.

As is clearly shown, no significant differences were observed in the amounts of fatty acids incorporated into total lipids per gram of rat liver.

Despite these similar incorporations significant differences were observed in the amounts of these acids incorporated into the major lipid fractions as is summarized in Table II. These data show the relatively low incorporation of 1-^{14}C-arachidonic acid into 1,2-diacylglycerol in contrast with the higher per cent of this acid observed in the phospholipid fraction.

The positional distribution of 1-^{14}C linoleic acid, 1-^{14}C-α-linolenic acid and 1-^{14}C-arachidonic acid in 3-*sn*-phosphatidylcholine and 3-*sn*-phosphatidylethanolamine is given in Table III. These results indicate a significantly higher incorporation of 1-^{14}C-arachidonic acid into the 2 position of this phospholipid fractions and the significant amount of 1-^{14}C-α-linolenic acid esterified into the 1 position in agreement with the results obtained by Possmayer et al. (2).

DISCUSSION

The activity of the enzymes leading to the biosynthesis of phospholipids and neutral lipids in vivo is under the control of regulatory mechanisms which are very difficult to manage in in vitro systems.

On the other hand the present conditions probably supply a continuous labeling of the fatty acid CoA derivative, immediate substrate for esterification. Nevertheless it is necessary to point out that the amount of labeled fatty acids incorporated into lipids depends either on their dilution during their transport in the blood or during their integration into the liver acyl-CoA pool.

Despite these facts the capacity of rat liver to esterify fatty acids is similar for the three substrates tested although the amounts of each substrate esterified into different esters are different. While no signficant differences were observed in the amounts of 1-^{14}C-linoleic and 1-^{14}C-α-linolenic acids incorporated into 1,2-diacylglycerol fractions, about one fifth as much 1-^{14}C-arachidonic acid was esterified in this fraction (Table II). Some differences were also observed in the phosphatides.

The relatively larger amount of 1-^{14}C-arachidonic acid in the triacylglycerol fraction compared to the diacylglycerol fraction suggests either a preferential esterification of the free position of 1,2-diacylglycerol by this acid or the selective acylation by the enzyme acyl-CoA-1,2-diacylglycerol-0-transferase in certain diglyceride units (9). The former assumption has been recently supported by in vitro experiments by DeKruyff et al. (15).

According to Kennedy (16) the principal biosynthetic route to 3-*sn*-phosphatidylcholine utilizes diglyceride as intermediate. While the 1-^{14}C linoleic acid incorporated into lecithin remains lower than in the 1,2-diacylglycerol fraction, about ten times more 1-^{14}C-arachidonic acid was found esterifying this phospholipid fraction. This finding suggests that the fatty acid redistribution by the deacylation-reacylation cycle (17-19) could account for this difference. This assumption is further supported by additional results (to be published) on the simultaneous in vivo incorporation of ^{3}H choline and 1-^{14}C-linoleic acid or 1-^{14}C-arachidonic acid into rat liver lecithin. While no significant differences were observed in the amounts of ^{3}H choline incorporated into the lecithin fraction per gram of rat liver, three times more 1-^{14}C-arachidonic acid than 1-^{14}C-linoleic acid were found esterifying these fractions.

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Regulation of Ovarian Cholesterol Esters: Evidence for the Enzymatic Sites of Prostaglandin-Induced Loss of Corpus Luteum Function[1]

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ABSTRACT

The regulation of cholesterol ester synthetase and cholesterol esterase by prostaglandins and gonadotrophins in luteinized ovaries of the rat was studied. Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) depressed ovarian cholesterol esters by 75% ($p<.025$) within 48 hr. Hypophysectomy (APX) produced a similar effect; prolactin administration to this group maintained cholesterol esters at a higher level than in the APX group but the trophic effect of prolactin was abolished by simultaneous $PGF_{2\alpha}$ treatment. Progesterone output in incubated ovarian slices was reduced 50% by $PGF_{2\alpha}$ treatment in vivo ($p<.005$), an effect similar to that produced by APX. Prolactin administration in vivo maintained the ability of the incubated tissue to synthesize progesterone at an elevated rate in APX rats but simultaneous $PGF_{2\alpha}$ treatment abolished this action of prolactin. Cholesterol ester synthetase activity was severely depressed ($p<.005$) by $PGF_{2\alpha}$ treatment to animals with intact pituitaries, a decrease similar to that produced by APX alone. The effect of APX on synthetase activity was reversed by prolactin treatment but not when $PGF_{2\alpha}$ was administered with prolactin. Esterase activity, also maintained by prolactin in APX animals ($p<.005$), was not affected to the same extent by $PGF_{2\alpha}$ although a decrease in activity was produced in both the intact and the APX + prolactin group by $PGF_{2\alpha}$ ($p<.10$). However simultaneous administration of luteinizing hormone (LH) reversed the effect of $PGF_{2\alpha}$ in the APX + prolactin + $PGF_{2\alpha}$ group on esterase activity. These data indicate that the luteolytic action of $PGF_{2\alpha}$ is directly on the corpus luteum and this action appears to be mediated by a neutralization of prolactin activity. The loss in synthetase activity and to some extent in esterase activity, induced by $PGF_{2\alpha}$ depressed ovarian cholesterol ester turnover and the availability of cholesterol for conversion to progesterone.

INTRODUCTION

The ovary is known to be dependent on pituitary hormones for continued function, and knowledge of the hormonal factors involved in the formation and maintenance of the corpus luteum is just emerging. In the rat for example, luteinizing hormone (LH) is known to increase the rate of progesterone biosynthesis when administered in vivo or when added directly to incubated slices of luteal tissue (1). Prolactin on the other hand has little acute effect on the rate of steroidogenesis (2) but is known to maintain the function and structure of the corpus luteum in hypophysectomized rats (3,4). In addition there is now considerable evidence that uterine factors may be present which under certain conditions hasten degeneration of the corpus luteum in the rat (5,6). It thus appears that both LH and prolactin as well as uterine factors are involved in regulating function of the rat corpus luteum but the mechanisms by which these substances exert their control is poorly understood.

The major role of the corpus luteum is the biosynthesis of progesterone. An understanding of the control mechanisms involved in the formation of this product is necessary in order to understand the regulation of luteal function. Claesson and Hillarp (7) suggested that cholesterol plays an important role in ovarian steroidogenesis by serving as a precursor reservoir since gonadotrophin treatment reduced the tissue levels of this substrate. Later workers (8) showed the specific decrease of cholesterol esters and Armstrong (9) further correlated ovarian cholesterol ester depletion with stimulation of steroidogenesis by LH. Behrman and Armstrong (10) demonstrated that LH increased the specific activity of cholesterol esterase (E.C.3.1.1.13) within an hour of in vivo administration, indicating the possibility of enzyme activation. Cholesterol ester depletion by LH was shown by Behrman et al. (11) to be a direct effect of LH and not a consequence of

[1]One of three papers being published from the Symposium, "Cholesteryl Esters," AOCS Meeting, Houston, May 1971.

TABLE I

Effect of Prostaglandin $F_{2\alpha}$ on Tissue Sterol Levels in Luteinized Ovaries

Treatment[a]	n	Cholesterol ester, mg/g[b]	Cholesterol, mg/g
Intact	5	11.0 ± 2.4[b■]	4.0 ± 1.1
Intact + $PGF_{2\alpha}$	5	3.1 ± 0.6[b●▲]	3.6 ± 0.9
APX	5	2.3 ± 0.1[b●▲]	3.2 ± 0.3
APX + PL	5	4.3 ± 1.3[b●♦]	3.8 ± 0.8
APX + PL + $PGF_{2\alpha}$	5	1.7 ± 0.4[b●▲]	3.5 ± 1.0
APX + PL + LH + $PGF_{2\alpha}$	3	2.0 ± 0.2[b●]	1.9 ± 0.1

[a]Abbreviations: $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; APX, hypophysectomy; PL, prolactin; LH, luteinizing hormone.

[b]b■ is significantly different from b● ($p<.025$); b▲ is significantly different from b♦ ($p<.10$).

increased steroidogenesis since inhibition of cholesterol oxidation with aminoglutethimide did not prevent the action of LH to reduce cholesterol esters. Neutralization of endogenous LH with specific antibodies (A/S) was shown by Behrman et al. (12) to result in an almost complete inhibition of cholesterol esterase activity within 24 hr. This was reflected in a large accumulation of ovarian cholesterol esters and a decrease in serum progesterone levels. Addition of LH A/S directly to incubated slices of ovarian tissue reduced progesterone biosynthesis. Thus in experiments where LH was injected or when circulating LH was removed by antibody neutralization, cholesterol esterase levels varied directly with the presence or absence of LH. These data support the hypothesis that activation of cholesterol esterase action is in part responsible for the increased steroidogenesis produced by acute LH treatment.

Prolactin is well recognized to be involved in maintaining luteal function and Wiest et al. (13) have shown that prolactin prevents the appearance of 20α-hydroxysteroid dehydrogenase. This enzyme catalyzes the reduction of progesterone to a progestationally inactive form 20α-hydroxy-pregn-4-en-3-one (20α-ol). Armstrong et al. (14) have shown that prolactin maintains ovarian cholesterol esters in rats lacking pituitary and under these conditions LH stimulation of steroidogenesis could be elicited. Behrman et al. (15) later demonstrated a specific action of prolactin to maintain high levels of sterol acyl transferase (E.C.3.1.2.2; cholesterol ester synthetase) and cholesterol esterase in hypophysectomized rats. The appearance of these enzymes was associated with corpus luteum formation and was directly correlated to increased levels of ovarian cholesterol esters.

Recently interest has greatly increased in the actions of prostaglandins in reproductive physi-

TABLE II

Effect of Prostaglandin $F_{2\alpha}$ on Luteal Cholesterol Ester Synthetase and Cholesterol Esterase Activity

Treatment[a]	n	Cholesterol ester synthetase CE cpm/min/mg protein[b]	Cholesterol esterase % Hydrolysis/min/mg protein[b]
Experiment 1			
Intact	4	855 ± 85[b■]	0.18 ± 0.03[b■▲]
Intact + $PGF_{2\alpha}$	4	234 ± 10[b●]	0.10 ± 0.02[b♦]
APX	4	119 ± 11[b●]	0.06 ± 0.02[b●]
APX + PL	4	544 ± 87[b■]	0.15 ± 0.01[b■▲]
APX + PL + $PGF_{2\alpha}$	4	160 ± 10[b●]	0.11 ± 0.02[b■♦]
APX + PL + LH + $PGF_{2\alpha}$	2	318 ± 21[b●]	0.14 ± 0.01[b■]
Experiment 2			
APX	4	65 ± 33[b■]	0.18 ± 0.01[b■]
APX + PL	3	1868 ± 156[b●]	0.43 ± 0.05[b●]
APX + PL + LH + $PGF_{2\alpha}$	4	316 ± 54[b■]	0.33 ± 0.05[b●]

[a]Abbreviations: See Table I.

[b]b■ is significantly different from b● ($p<.005$); b▲ is significantly different from b♦ ($p<.10$).

TABLE III

Effect of Prostaglandin $F_{2\alpha}$ on Progestin Output in Incubated Luteal Tissue

Treatment[a]	n	Progesterone $\mu g/g$[b]	20α-o1, $\mu g/g$[b]
Intact	5	$57.5 \pm 7.6^{b\blacksquare}$	$40.0 \pm 10.1^{b\blacklozenge}$
Intact + $PGF_{2\alpha}$	5	$23.3 \pm 2.8^{b\bullet}$	38.2 ± 5.6
APX	5	$28.5 \pm 6.7^{b\bullet\blacktriangle}$	$18.2 \pm 2.8^{b\blacklozenge}$
APX + PL	5	$38.4 \pm 4.4^{b\blacklozenge}$	$46.6 \pm 13.6^{b\blacklozenge}$
APX + PL + $PGF_{2\alpha}$	5	$11.6 \pm 1.8^{b\blacklozenge}$	34.2 ± 7.0
APX + PL + LH + $PGF_{2\alpha}$	3	14.5 ± 4.3	38.3 ± 6.5

[a]Abbreviations: See Table I.

[b]$b\blacksquare$ is significantly different from $b\bullet$ ($p<.005$); $b\blacktriangle$ is significantly different from $b\blacklozenge$ ($p<.10$); $b\blacklozenge$ is significantly different from $b\blacklozenge$ ($p<.001$).

ology. These compounds appear to be involved in many hormone-mediated processes (16) and show a pronounced degenerative effect on the corpus luteum of the rat (17) and many other species (18,19,20). Behrman et al. (21) have shown that prostaglandin administration causes an abrupt decrease in ovarian progesterone secretion in the pregnant and pseudopregnant rat. The mechanism of the luteolytic action of prostaglandin is not completely known although Pharris (22) has suggested that prostaglandin may decrease the blood flow to the ovary. However Behrman et al. showed that the ovarian venous flow rate was unaffected during prostaglandin treatment (21) or several hours later (23), although a marked decrease in progesterone secretion had occurred.

The present experiments evaluate the effect of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) on corpus luteum function and are designed to obtain information on the site of action of prostaglandin in this gland. The effect of prostaglandin treatment in vivo on the enzymes involved in cholesterol ester turnover was examined and correlated with tissue levels of cholesterol ester and ovarian progesterone output in incubated ovaries from intact rats and hypophysectomized rats treated with gonadotrophins.

MATERIALS AND METHODS

Animals

Immature female rats purchased from the Holtzman Co., Madison, Wis. were housed in temperature-controlled quarters which provided 14 hr of light and 10 hr of darkness per day. Food and water were given ad libitum. At 31 days of age the animals received 50 IU of Pregnant Mare Serum (PMS) followed 65-70 hr later with 25 IU of Human Chorionic Gonadotrophin (HCG) in order to induce highly luteinized ovaries. Three days after HCG treatment hypophysectomy was carried out as described earlier (15). At autopsy the sella turcicae were examined with a loupe and ovarian tissues were discarded if the animal was not completely hypophysectomized.

Treatment Schedule

All treatments began immediately following hypophysectomy, were administered by the subcutaneous route, and were continued for two days. Animals were sacrificed 12-14 hr following the last treatment. Prostaglandin $F_{2\alpha}$ was dissolved in 10% ethanol-saline-0.01% Na_2CO_3 and then mixed in a saline-gelatin solution to give a final concentration of 1 mg/ml in 15% gelatin. $PGF_{2\alpha}$ was administered at a dosage of 0.5 mg/kg b.i.d. Prolactin (NIH-P-S9) was administered at a dosage of 2 IU b.i.d. in 15% gelatin and LH (NIH-LH-S16) at a dosage of 10 μg b.i.d. in 15% gelatin.

Preparation of Tissues

Ovarian tissue from animals within a treatment group were pooled. Portions from each ovary were separated into two subpools, one for incubation of ovarian tissue slices and one for assay of cholesterol esterase and cholesterol ester synthetase activity. The data on steroidogenesis in vitro were obtained from two separate experiments which contained 2 and 3 replicates respecitvely. The enzyme analyses were obtained from 2 replicates in each experiment. Prior to incubation the weight of the ovarian slices in each flask was recorded. The enzymes were partially purified by centrifugation of ovarian tissue homogenized (75 mg/ml) with 0.25 M sucrose containing 0.001 M EDTA. The first centrifugation was at 10,000 rpm for 15 min and the second at 100,000 x g for 60 min.

Enzyme Assays

Cholesterol esterase activity was assayed in the soluble fraction and cholesterol ester synthetase activity in the pellet fraction after centrifugation at 100,000 x g. Enzyme activity was measured at initial rates by incubating radio-labeled substrates with the respective fractions at 37 C for 3 and 6 min as described earlier (15). The data are expressed as % hydrolysis/min or cholesterol ester cpm/min per mg of protein rather than in the standard enzyme units, since endogenous substrates were present. However because of the centrifugation procedures employed and the care taken to remove the subcellular fractions free of contamination from the lipid layer, the endogenous substrate level is minimal and constant as shown earlier (10).

Incubation Procedure

Ovarian tissue slices were prepared and incubated in 4 ml Krebs-Ringer bicarbonate buffer containing 1 mg/ml of glucose, for 2 hr at 37 C in an atmosphere at 95% O_2-5% CO_2. Each incubation flask contained 150-250 mg of tissue and the incubation was stopped by placing the flasks on dry ice and then storing at -20 C until extraction and analysis.

Analytical Methods

Incubated tissues were homogenized in the surrounding media to which was added tracer amounts of cholesterol-1,2-^{3}H, cholesterol-7α-^{3}H palmitate, progesterone-1,2-^{3}H, and 20α-ol 1,2-^{3}H (purchased from New England Nuclear Corp., Boston). Tracers were purified by thin layer chromatography (TLC) and used to correct for losses during extraction and chromatography. The homogenates were extracted first with 4 volumes and second with 3 volumes of diethyl ether. The ether extract was dried under a stream of nitrogen and the lipids were separated by TLC in a solvent system composed of hexane-ether-acetic acid 75:25:2. The sterol fractions were identified from standards run in parallel, removed, extracted from the silica gel using chloroform-MeOH 1:1 and quantitated using a fluorescent technique described earlier (10). The combined steroid fractions were removed eluted and separated by TLC using methylene chloride-ether 5:2, eluted from the silica gel and quantitated by gas liquid chromatography as described earlier (11). Protein was determined by the method of Lowry et al. (24).

RESULTS

The effect of prostaglandin treatment on ovarian sterols in animals with an intact pituitary gland and in hypophysectomized-gonadotrophin-treated animals is shown in Table I. In animals with an intact pituitary, $PGF_{2\alpha}$ produced a highly significant 75% decrease in the ovarian content of cholesterol esters which was equivalent to that produced by hypophysectomy alone. Prolactin treatment maintained cholesterol esters at about twice the level seen in the hypophysectomized control group. When $PGF_{2\alpha}$ was administered with prolactin a loss in the trophic action of prolactin to maintain ovarian cholesterol esters occurred. Simultaneous administration of LH with prolactin did not prevent this action of $PGF_{2\alpha}$ in hypophysectomized animals. Ovarian levels of free cholesterol were not affected by treatment, with the possible exception of the hypophysectomized group treated with LH, prolactin and $PGF_{2\alpha}$, where a decline of about 50% was observed when compared to the other groups.

In Table II, Experiment 1, the effect of identical treatments on the specific activity of cholesterol ester synthetase and cholesterol esterase is shown. Prostaglandin depressed synthetase activity (75%) when administered to intact animals. Hypophysectomy also reduced synthetase activity to a similar extent and this effect was due to a loss of circulating gonadotrophin since replacement with prolactin maintained enzyme activity. In previous studies (15) we demonstrated that LH elicited no trophic action to maintain enzyme activity in hypophysectomized animals and a combination of LH and prolactin was no more effective than prolactin alone. In fact, with respect to synthetase activity, LH administration with prolactin was less effective than prolactin alone. The effect of prostaglandin on synthetase activity was a direct action on the ovary, a conclusion which is supported by the observed loss of enzyme activity in hypophysectomized animals treated with prolactin and $PGF_{2\alpha}$. Concomitant administration of LH and prolactin did not prevent the lesion induced at the level of the synthetase enzyme by $PGF_{2\alpha}$. Cholesterol esterase activity, although markedly reduced by hypophysectomy (-70%), was depressed to a smaller extent in intact rats than was synthetase activity by $PGF_{2\alpha}$ administration. Prolactin maintained high levels of esterase activity in hypophysectomized animals and $PGF_{2\alpha}$ administration to this group also produced a moderate decline in enzyme activity but again not as severe as the effect produced on synthetase activity. Concomitant administration of LH and prolactin was able to completely neutralize the effect of $PGF_{2\alpha}$ on esterase activity. In an additional experiment with hypophysectomized

animals, prolactin was shown to increase both synthetase and esterase activity very markedly, Table II, Experiment 2. However $PGF_{2\alpha}$, while almost completely preventing the trophic action of prolactin on synthetase activity, again did not significantly reduce esterase activity in the presence of prolactin and LH.

The effect of $PGF_{2\alpha}$ administered in vivo on the progesterone and 20α-ol levels in incubated ovarian slice preparations is shown in Table III. The level of progesterone output can be directly correlated in these experiments with ovarian cholesterol ester output and cholesterol ester synthetase activity. For example progesterone was significantly reduced (-50%) by $PGF_{2\alpha}$ administration to animals with an intact pituitary and by hypophysectomy. Prolactin replacement increased progesterone output in hypophysectomized animals but again $PGF_{2\alpha}$ prevented the trophic action of prolactin administered alone or in combination with LH. Indeed progesterone output was reduced even below the hypophysectomized group when $PGF_{2\alpha}$ was administered with prolactin in hypophysectomized animals. The output of 20α-ol did not follow a pattern similar to that of progesterone with these treatments. Prostaglandin did not reduce 20α-ol when administered to animals with an intact pituitary and thus the significant reduction in total progestins (progesterone + 20α-ol) observed with this treatment was due to a selective decrease in progesterone. However hypophysectomy did reduce 20α-ol output (-50%) and the significant decline noted for the total progestin level (-50%) arose from a decrease in both progesterone and 20α-ol. Prolactin treatment prevented the detrimental effect of hypophysectomy on 20α-ol output but $PGF_{2\alpha}$ did not interfere with the trophic action of prolactin in this case as it had with progesterone output. In general the changes in the level of total progestins reflected the effect of treatment on progesterone level except for the hypophysectomy and prolactin replacement groups.

DISCUSSION

The results of studies reported herein indicate that prostaglandin produced a lesion in the luteal cell which prevented adequate storage of cholesterol esters and impaired the ability of the corpus luteum to produce progesterone. This conclusion is supported by the closely associated effects of treatments on cholesterol ester synthetase activity, tissue levels of cholesterol ester and progesterone output. The site of the prostaglandin-induced lesion appeared to be located primarily at the level of cholesterol ester synthesis as the synthetase enzyme was more severely affected than was the hydrolytic enzyme. The mechanism by which prostaglandin impaired the ability of the corpus luteum to turn over cholesterol ester appeared to arise from a loss in the trophic expression of prolactin. In previous studies (15) we have shown that prolactin specifically maintains cholesterol ester synthetase, cholesterol esterase activity and tissue cholesterol esters in the corpus luteum of the hypophysectomized rat. Others have documented the ability of prolactin to maintain progesterone secretion (14) which was confirmed in the present experiments.

Although $PGF_{2\alpha}$ completely neutralized the trophic effect of prolactin on cholesterol ester synthetase activity, its effect was not as marked on esterase activity and indeed it had no effect on esterase activity when LH and prolactin were administered together. Pharriss (22) has previously demonstrated the ability of $PGF_{2\alpha}$ to neutralize the trophic action of prolactin to maintain elevated ovarian progesterone levels in hypophysectomized rats. In earlier studies we demonstrated that $PGF_{2\alpha}$ reduced ovarian progesterone secretion within 30 min of an iv injection but found that it did not affect ovarian venous flow rate (21). In experiments where LH was administered simultaneously with $PGF_{2\alpha}$, the decline in progesterone secretion was prevented. These observations on the acute effect of $PGF_{2\alpha}$ may not be directly comparable to the data in the present experiments where $PGF_{2\alpha}$ was administered chronically, but it is of interest to note that LH, when administered with prolactin, was able to prevent any change by $PGF_{2\alpha}$ on esterase activity. In addition LH administration along with prolactin appeared to be partially effective in maintaining a higher synthetase activity in the presence of prostaglandin compared to that of prolactin alone. Thus it is possible that $PGF_{2\alpha}$ may reduce esterase activity which decreases the amount of cholesterol available for steroidogenesis and causes a decline in progesterone output. However the decrease in progesterone output appeared to be more closely related to a loss in synthetase activity induced by $PGF_{2\alpha}$ which led to a decrease in cholesterol esters and thereby reduced the amount of cholesterol available for conversion to progesterone.

Prostaglandin added directly to incubated slices of ovarian tissue has been shown by us (23) and by others (25) to stimulate steroidogenesis; yet we have also observed that progesterone secretion is reduced within minutes following an iv injection of $PGF_{2\alpha}$ (21). The opposite effect of prostaglandin in vitro and in vivo is puzzling and no adequate explanation is

currently available. However we have observed (12) that cholesterol may be more accessible for steroidogenesis in tissue incubated in vitro than in situ and the possibility exists that $PGF_{2\alpha}$ may in some unknown manner increase the availability of cholesterol for steroidogenesis in vitro.

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Response of Rat Adrenal Cholesteryl Esters to Cold Stress[1]

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ABSTRACT

Male rats were maintained on diets containing corn oil or hydrogenated coconut oil. The compositions of the adrenal cholesteryl esters were determined in control animals and in those subjected to cold stress (4 C for 30 min). Total sterol ester content was lower in the stressed rats. In those receiving corn oil there were selective decreases in 16:1, 18:2 and 20:4(*n*-6) esters but not in 22:4(*n*-6). In the coconut oil fed, essential fatty acid (EFA) deficient animals, the decreases in 20:4(*n*-6) and 22:4(*n*-6) were quite selective, but the concentrations of the cholesteryl esters of 20:3(*n*-9) and 22:3(*n*-9) were also selectively reduced in the stressed animals. Olive oil and corn oil-ethyl erucate were employed as dietary fats in a second experiment. Plasma corticosterone was lower in animal fed ethyl erucate and subjected to cold stress. Cholesteryl erucate was the major adrenal ester in animals receiving dietary erucate but it was not well utilized in animals subjected to stress. The cholesteryl esters of linoleate and arachidonate were preferentially utilized in both of these dietary groups. Cholesteryl arachidonate was selectively utilized in all four dietary groups studied and may constitute the preferred substrate for rat adrenal cholesteryl ester hydrolase.

INTRODUCTION

The rat adrenal contains more esterified cholesterol in proportion to its weight than any other tissue in the body. Cholesteryl esters account for about 4-6% of the wet weight of this tissue (1), far more than that found in plasma and liver (ca. 0.05%) (2) or even the 1-1.5% occurring in the ovary (3), the only other tissue which is comparable to the adrenal in this respect. Moreover rat adrenal cholesteryl esters have a somewhat unusual fatty acid composition; not only are they rich in polyunsaturated acids but a major component under normal dietary conditions is a docosatetraenoic acid. This acid was isolated from canine adrenal lipids by Chang and Sweeley (4) who characterized it as the 7,10,13,16-isomer, 22:4(*n*-6). Although the adrenal lipids from several species contain this acid (5), the levels are considerably lower than those found in the rat tissue. The rat adrenal is not unique in containing high concentrations of docostatetraenoic acid however, since ovarian cholesteryl esters from this species also contain this acid in abundance (6). In both of these tissues the docosatetraenoic acid occurs primarily in the cholesteryl ester fraction with very little being found in the other lipid fractions. The occurrence of C22-unsaturated acids in the rat adrenal is not limited to 22:4(*n*-6) and the tissue lipids do respond to dietary manipulation. Thus in essential fatty acid deficiency 22:3(*n*-9) is a major constituent of this tissue (7-9), and dietary erucic acid is deposited extensively in the adrenal (1). Egwim and Sgoutas (10) have reported that feeding hydrogenated soybean oil not only results in the deposition of elaidic acid but also of 9,13-docosadienoic acid in rat adrenal lipids. This latter acid is unusual since it does not contain the classic "methylene interrupted" double bond structure of most naturally occurring unsaturated acids. In all of the above instances the docosenoic acids were deposited primarily as cholesteryl esters and their deposition was accompanied by an increase in the total cholesteryl ester content of the tissue, suggesting additional deposition of the unusual ester rather than a simple substitution for other esters. Recent studies in our laboratory have confirmed the similarity between rat adrenal and ovary with respect to the deposition of 22:3(*n*-9) (6) and 22:1(*n*-9) (Walker, unpublished data) in the cholesteryl ester fraction under the appropriate dietary conditions.

Because of the high concentrations of polyunsaturated acids of the (*n*-6)-series in rat adrenal cholesteryl esters, several workers have investigated the function of these compounds. It is known that the cholesteryl ester fraction of the adrenal decreases in conditions resulting in synthesis of steroid hormones, although the free sterol fraction does not appear to change significantly (11). The esterified cholesterol thus appears to be functioning as a reservoir of sterol for hormone biosynthesis. The speculation of Sinclair (12) that specific esters, namely those of the (*n*-6)-polyunsaturated acids, may act as precursors for steroid hormone production is not unreasonable. The work of Haya-

[1]One of three papers being published from the Symposium, "Cholesteryl Esters," AOCS Meeting, Houston, May 1971.

TABLE I

Fatty Acid Composition of Dietary Fats[a]

Acid	Weight, %			
	Corn oil	Hydrogenated coconut oil	Olive oil	Corn oil-ethyl erucate
8:0	---	6.6	---	-----
10:0	---	7.2	---	---
12:0	---	52.6	---	---
14:0	---	17.1	---	---
16:0	11.7	7.5	11.1	2.3
18:0	2.3	8.6	2.7	0.4
18:1	29.5	0.3	76.6	5.6
18:2	55.5	⟨0.1	7.9	10.4
22:1	---	---	---	81.3

[a]Minor constituents omitted from table.

shida and Portman (13) demonstrating the impaired ability of adrenal tissue from essential fatty acid deficient rats to synthesize corticosteroids in vitro lends credence to this thesis. In a comprehensive but apparently little publicized series of studies, a group of workers from the University of Kyoto Medical School (8,14-16) investigated the effects of stress on adrenocortical function in normal, EFA deficient and vitamin B_6 deficient rats. The resting urinary formaldehydogenic corticoid and plasma fluorometric corticoid levels were significantly lower in EFA deficient rats and these animals exhibited a marked inability to respond to such stressful situations as adrenocorticotrophic hormone (ACTH) injection, cold stress, surgical insult, fracture of the ulna and radius or starvation, when compared with animals receiving adequate levels of dietary linoleate (14). Fukuda (15) also reported a parallel relationship between serum EFA levels and adrenocortical capacity in human surgical patients. Muraoka (8) extended the work in rats by demonstrating that there was a preferential decrease in adrenal cholesteryl arachidonate in response to ACTH injection. A similar finding was reported by Gidez and Feller (17) in rats subjected to unilateral adrenalectomy and they also found that oleate and linoleate were undergoing preferential utilization in addition to the arachidonate.

It is apparent that cholesteryl esters of the essential fatty acids, particularly arachidonic acid, are mobilized during glucocorticoid synthesis by the rat adrenal. However a number of questions remain unanswered in the above reports. The function of the high concentration of cholesteryl adrenate has not been determined, nor has it been determined that the impaired adrenocortical function in EFA deficient rats is due to the presence of 20:3(*n*-9) and 22:3(*n*-9) in the adrenals in place of 20:4(*n*-6) and 22:4(*n*-6). In the studies of Muraoka (8) substantial amounts of the latter acids were present in the adrenal cholesteryl esters of the deficient animals, but unfortunately expression of the data in terms of per cent of total fatty acids renders assessment of the absolute changes in the concentrations of 20:3(*n*-9) and 22:3(*n*-9) difficult. Finally the deposition of large amounts of the somewhat atypical erucic acid (1), partly at the expense of the (*n*-6)-polyunsaturated acids, has not been considered in terms of utilization of such a cholesteryl ester for corticosteroid production. The following experiments were therefore undertaken in an attempt to provide answers to these questions.

MATERIALS AND METHODS

Male weanling rats of the Wistar strain were purchased from Woodlyn Farms, Guelph, Ontario. Dextrose was purchased from Canada Starch Co., Toronto, Ontario, cellulose (alpha floc) from Lee Chemicals Ltd., Toronto, Ontario, and vitamin free casein, the mineral mix (modified Williams-Briggs formulation) and vitamins from General Biochemical Corp., Chagrin Falls, Ohio. A vitamin mix was prepared containing the following ingredients per kilogram diet: vitamin A (palmitate ester), 24,000 IU; vitamin D (on bran carrier), 4,000 IU; dl α-tocopherol acetate, 120.0 mg; menadione, 1.0 mg; thiamine hydrochloride, 10.0 mg; pyridoxine hydrochloride, 5.0 mg; niacin, 50.0 mg; calcium pantothenate, 20.0 mg; *p*-aminobenzoic acid, 20.0 mg; riboflavin, 10.0 mg; folic acid, 1.0 mg; biotin 0.5 mg; vitamin B_{12} (as 0.1% trituration with mannitol), 10.0 mcg; inositol, 1.0 g; choline chloride, 1.0 g. Dextrose was employed as a carrier in sufficient quantity that the above vitamin requirements were met by the addition of 1% vitamin mix to

TABLE II

Effect of Cold Stress on the Cholesteryl Esters From the Adrenals of Rats Fed Corn Oil

Ester	Control (5)[a]		Stressed (5)		Decrease μmoles/g tissue	SI[b]
	μmoles/g tissue	Mole %	μmoles/g tissue	Mole %		
14:0	1.8 ± 0.4	2.5	1.3 ± 0.3	2.6	0.5	0.9
16:0	9.1 ± 1.1	12.6	6.8 ± 0.8	13.5	2.3	0.8
16:1	3.8 ± 1.0	5.2	1.8 ± 0.3	3.6	2.0	1.7
18:0	2.3 ± 0.4	3.2	2.7 ± 0.6	5.4	-0.4	---
18:1	13.1 ± 1.7	18.0	8.8 ± 1.5	17.5	4.3	1.1
18:2	5.2 ± 0.7	7.2	3.0 ± 0.2	6.0	2.2	1.4[c]
20:4(*n*-6)	15.4 ± 2.4	21.2	9.7 ± 1.2	19.3	5.7	1.2[c]
22:4(*n*-6)	15.5 ± 2.8	21.4	11.2 ± 1.0	22.3	4.3	0.9
Total	72.5		50.3		22.2	

[a]Mean±SEM, number of samples in parentheses. Minor components omitted from table.

[b]Selectivity Index = decrease in concentration of ester expressed as per cent of decrease in total ester divided by mole per cent of ester in control (17). A value greater than unity indicates a preferential loss of the ester in the stressed animal.

[c]$p<0.05$

the diet.

Corn oil (Mazola brand) and olive oil (Unico brand) were purchased locally, hydrogenated coconut oil and erucic acid were generously donated by Canada Packers Ltd., Toronto, Ontario. The erucic acid was esterified prior to use by refluxing with 2% H_2SO_4 in ethanol. The fatty acid compositions of the dietary fats are presented in Table I.

The animals were maintained on semisynthetic diets containing dextrose (59%), vitamin free casein (20%), cellulose (5%), fat (10%), minerals (5%) and vitamin mix (1%). In the initial experiment the diets contained either corn oil or hydrogenated coconut oil and animals were sacrificed after 25 weeks on these diets. The fats employed in the second experiment were olive oil and a mixture of corn oil and ethyl erucate (1:5 w/w), both included in the diet at the 12% level, the extra 2% fat substituting for carbohydrate calories. This experiment was terminated after 10 weeks on the experimental diets.

The animals on each diet were randomly divided into two groups at the termination of the experiment. One group was sacrificed immediately by anaesthetizing with sodium pentabarbital and exsanguinating via the abdominal aorta using a heparinized syringe. The blood was retained for plasma corticosteroid assay if required and the adrenals were rapidly removed and chilled in liquid nitrogen. The second group of animals was subjected to cold stress by immersing in water at 4 C for 30 min prior to sacrifice by the procedure outlined above. Blood and adrenals were obtained as before.

Within 15 min of sacrifice of the rat the blood was centrifuged at 5000 g for 15 min at 4 C and the plasma was isolated. Plasma corticosterone was assayed by the procedure of Martin and Martin (18). The two adrenals from each rat were carefully freed of perirenal fat, extruded from the capsules and weighed. They were then homogenized (Polytron, model PT 10, Brinkman Instruments Ltd., Rexdale, Ontario) for 30 sec in 10 ml of 2:1 chloroform-methanol and extraction of the lipid continued for 60 min at room temperature under nitrogen. After removal of the residue by filtration the extract was washed with water and the lipids isolated as previously described (19). Total lipids were fractionated by thin layer chromatography (TLC) on Adsorbosil 1 (Applied Science Labs. Inc., State College, Pennsylvania) with petroleum ether-diethyl ether (97:3 v/v) as developing solvent. Cholesteryl esters, visualized by spraying the plate with Rhodamine 6G and viewing under ultra violet light, were extracted from the gel with freshly redistilled diethyl ether. Methyl pentadecanoate was added as an internal standard, the cholesteryl esters were transesterified with boron fluoride-methanol and the resulting methyl esters purified by TLC (19). The fatty acid composition of the adrenal cholesteryl esters was determined by gas liquid chromatography using the equipment and procedures previously described (19). The esters from animals fed corn or coconut oil were fractionated on a 150 x 0.2 cm column containing 15% EGSS-X on 80-100 mesh Chromosorb W (Applied Science Labs., Inc.) and operated isothermally at 180-185 C. In order to separate 18:3(*n*-3) from 20:1(*n*-9) and

TABLE III

Effect of Cold Stress on the Cholesteryl Esters From the Adrenals of Rats Fed Hydrogenated Coconut Oil

Ester	Control (5)[a]		Stressed (5)		Decrease μmoles/g tissue	SI[b]
	μmoles/g tissue	Mole %	μmoles/g tissue	Mole %		
14:0	6.0 ± 0.5	6.9	6.2 ± 1.3	9.6	-0.2	---
16:0	10.6 ± 1.3	12.1	8.9 ± 1.2	13.8	1.7	0.6
16:1	4.3 ± 0.6	4.9	4.3 ± 0.4	6.6	0.0	---
18:0	3.8 ± 0.5	4.3	3.1 ± 0.3	4.8	0.7	0.7
18:1	17.3 ± 2.6	19.8	14.7 ± 2.9	22.7	2.6	0.6
18:2	1.1 ± 0.2	1.3	1.0 ± 0.2	1.5	0.1	0.3
20:3(*n*-9)	16.0 ± 1.5	18.3	9.9 ± 1.5	15.3	6.1	1.5[c]
20:4(*n*-6)	1.7 ± 0.3	1.9	0.5 ± 0.1	0.8	1.2	2.8[d]
22:3(*n*-9)	18.6 ± 2.1	21.3	11.4 ± 3.6	17.6	7.2	1.5
22:4(*n*-6)	2.1 ± 0.3	2.4	0.5 ± 0.1	0.8	1.6	2.9[d]
Total	87.4		64.7		22.7	

[a]Mean±SEM, number of samples in parentheses. Minor components omitted from table.

[b]Selectivity Index = decrease in concentration of ester expressed as per cent of decrease in total ester divided by mole per cent of ester in control (17). A value greater than unity indicates a preferential loss of the ester in the stressed animal.

[c]$p<0.05$.

[d]$p<0.01$.

20:4(*n*-6) from 22:1(*n*-9), a 300 x 0.2 cm column containing 3% EGSP-Z on 100-120 mesh Gaschrom Q (Applied Science Labs., Inc.) and operated isothermally at 175 C was employed in the separation of esters from animals on the olive oil and corn-erucic diets. The inclusion of the internal standard permitted the absolute concentrations of individual cholesteryl esters to be determined. The t-test (20) was employed in determining the significance of the differences between experimental groups.

RESULTS

Essential Fatty Acid Deficiency and Stress

Animals maintained on the hydrogenated coconut oil exhibited the classical symptoms of essential fatty acid deficiency including poor growth, scaliness of the tail and feet, and coarseness of fur. Arachidonic and 22:4(*n*-6) acids were major components of the adrenal cholesteryl esters from the rats receiving corn oil but constituted only a small fraction of the esters from the deficient animals (Table II and III). In the deficient rats the polyenoic acids of the (*n*-9)-series, 20:3 and 22:3, accounted for almost 40% of the total acids from this adrenal fraction. The total cholesteryl ester content was slightly higher in the deficient adrenals.

Subjecting the rats to cold stress resulted in a decrease in the total cholesteryl esters. Although decreases in the concentrations of total esters were similar for the normal and EFA deficient animals, a proportionately greater decrease was observed in rats receiving corn oil (30.6% vs. 25.9%). In animals maintained on corn oil (Table II) most esters were found to decrease in concentration. However only the changes in 18:2 and 20:4 were demonstrated to be significant ($p<0.05$) as a result of the cold stress. The selectivity index (SI) was computed in order to determine the relative utilization of the different esters. Both arachidonic and linoleic esters, which were significantly lower in the stressed rats, had selectivity indices in excess of unity. In addition the selectivity indices of oleic and palmitoleic esters were greater than unity. The index of 22:4(*n*-6) ester was only 0.9.

In rats maintained on hydrogenated coconut oil (Table III) the concentrations of the 22:4(*n*-6), 20:4(*n*-6) and 20:3(*n*-9) esters were significantly lowered as a result of cold stress, and all of these esters exhibited selectivity indices greater than 1.0. Although the SI of 22:3 (*n*-9) was the same as that of 20:3(*n*-9), the difference in concentrations of this ester in the control and stressed adrenals was not demonstrably significant.

Erucic Acid and Stress

The plasma corticosterone levels were determined in rats receiving olive oil or corn oil-ethyl erucate either in the resting state or subjected to stress. These data are presented in Table IV. Although the resting levels of plasma corticosterone were similar in the two dietary groups,

the concentration of the glucocorticoid was significantly greater in the stressed rats fed olive oil than in those receiving ethyl erucate and subjected to stress.

The compositions of the cholesteryl esters from animals receiving the olive oil and erucic acid containing diets are presented in Tables V and VI. The two dietary fats were quite similar in fatty acid composition except for the nature of the major monoenoic acid present, oleic in the case of the olive oil and erucic acid in the corn oil-ethyl erucate mixture (Table I). Olive oil contained more palmitic and stearic and slightly less linoleic acid than the other oil. The corn oil-ethyl erucate diet resulted in the deposition of large amounts of erucic acid in the adrenal cholesteryl esters (Table VI). It was in fact the major fatty acid component of this lipid fraction, accounting for almost 30 mole % of the total acids. Eicosenoic acid, 20:1(*n*-9), accounted for 8% of the total acids. However the occurrence of these two acids in the adrenal cholesteryl esters was not contingent on the presence of erucate in the diet, since both were found in the rats maintained on the olive oil diet. The rat adrenal is apparently capable of elongating oleic acid to 20:1(*n*-9) and 22:1(*n*-9) or of incorporating these acids from the plasma. The major differences between the olive oil and corn-erucate groups were the presence of large amounts of erucic acid and less oleic acid in the adrenals of the latter. The proportions of most of the other acids, including the (*n*-9)-acids, were slightly lower in the group receiving ethyl erucate, although the absolute concentrations were similar or slightly higher in some instances. Total cholesteryl ester content per gram wet tissue was greater in this group.

TABLE IV

Effect of Diet and Cold Stress on Plasma Corticosterone

	μg/100 ml	
	Olive oil	Corn oil-erucic
Control	9.9 ± 0.93[a]	9.9 ± 1.15
Stressed	61.3 ± 5.43	42.5 ± 6.51[b]

[a]Mean±SEM.
[b]$p<0.05$.

Cold stress resulted in significant decreases in the concentrations of the 16:1, 18:2, 20:1(*n*-9), 20:4(*n*-6) and 22:4(*n*-6) esters of cholesterol in rats receiving olive oil (Table V). The SI of all of these acids exceeded unity although the values for 20:1(*n*-9) and 22:4(*n*-6) were only slightly greater than 1.0. In this group the SI of myristic acid also exceeded unity. The decrease in cholesteryl ester content was 34.7% of the control value in the olive oil fed rats. In contrast, cold stress resulted in a decrease of only 16.8% in animals receiving the ethyl erucate diet, but this change was not significant at the 5% level. In only one instance, oleic acid, was a significant decrease noted in an individual ester in this group, although 14:0, 16:1, 18:1, 18:2 and 20:4 all had SI values greater than unity. A prominent feature of this group was the lack of utilization of cholesteryl

TABLE V

Effect of Cold Stress on the Cholesteryl Esters From the Adrenals of Rats Fed Olive Oil

Ester	Control (9)[a]		Stressed (7)		Decrease μmoles/g tissue	SI[b]
	μmoles/g tissue	Mole %	μmoles/g tissue	Mole %		
14:0	1.5 ± 0.2	2.9	0.7 ± 0.1	2.1	0.8	1.5
16:0	6.0 ± 0.6	11.5	4.8 ± 1.1	14.1	1.2	0.6
16:1	3.0 ± 0.3	5.8	1.6 ± 0.2	4.7	1.4	1.3[d]
18:0	1.8 ± 0.2	3.5	1.7 ± 0.5	5.0	0.1	0.2
18:1	14.8 ± 1.5	28.4	10.7 ± 1.4	31.5	4.1	0.8
18:2	2.2 ± 0.4	4.2	1.1 ± 0.2	3.2	1.1	1.5[c]
20:1(*n*-9)	2.6 ± 0.3	5.0	1.6 ± 0.2	4.7	1.0	1.1[c]
20:4(*n*-6)	5.1 ± 0.6	9.8	2.9 ± 0.3	8.5	2.2	1.2[d]
22:1(*n*-9)	1.2 ± 0.8	2.3	0.9 ± 0.2	2.6	0.3	0.7
22:4(*n*-6)	8.8 ± 1.5	16.9	5.5 ± 0.4	16.2	3.3	1.1[c]
Total	52.1		34.0		18.1	

[a]Mean±SEM, number of samples in parentheses. Minor components omitted from table.
[b]Selectivity Index = decrease in concentration of ester expressed as per cent of decrease in total ester divided by mole per cent of ester in control (17). A value greater than unity indicates a preferential loss of the ester in the stressed animal.
[c]$p<0.05$
[d]$p<0.01$.

TABLE VI

Effect of Cold Stress on the Cholesteryl Esters From the Adrenals of Rats Fed Corn Oil-Ethyl Erucate

Ester	Control (9)[a]		Stressed (7)		Decrease μmoles/g tissue	SI[b]
	μmoles/g tissue	Mole %	μmoles/g tissue	Mole %		
14:0	2.2± 0.1	3.2	1.3± 0.2	2.3	0.9	2.4
16:0	6.3± 0.4	9.2	5.3± 0.5	9.3	1.0	0.9
16:1	2.4± 0.1	3.5	1.9± 0.2	3.3	0.5	1.2
18:0	1.4± 0.1	2.0	1.3± 0.1	2.3	0.1	0.5
18:1	9.9± 0.5	14.5	7.6± 0.7	13.4	2.3	1.4[c]
18:2	1.0± 0.1	1.5	0.8± 0.1	1.4	0.2	1.1
20:1(*n*-9)	5.4± 0.5	7.9	4.5± 0.5	7.9	0.9	1.0
20:4(*n*-6)	6.2± 0.4	9.1	4.9± 0.5	8.6	1.3	1.2
22:1(*n*-9)	19.9± 2.1	29.1	18.3± 2.2	32.2	1.6	0.5
22:4(*n*-6)	10.0± 0.7	14.6	8.7± 0.8	15.3	1.3	0.8
Total	68.3		56.8		11.5	

[a]Mean±SEM, number of samples in parentheses. Minor components omitted from table.

[b]Selectivity Index = decrease in concentration of ester expressed as per cent of decrease in total ester divided by mole per cent of ester in control (17). A value greater than unity indicates a preferential loss of the ester in the stressed animal.

[c]$p < 0.05$.

erucate (SI = 0.5).

DISCUSSION

The total adrenal cholesteryl ester content was somewhat lower in the present study than in those reported previously. Gidez and Feller (17) found over 110 μmoles of esters per gram tissue in unstressed rats, whereas in the control animals receiving corn oil in the present study, the sterol ester level was only slightly more than 70 μmoles per gram tissue. Moreover in the second experiment of this study even lower levels of total cholesteryl esters were found. However the animals employed in this second experiment were considerably younger than those used in the EFA deficient study. As noted previously, feeding an EFA deficient diet (9) or one containing erucic acid (1) resulted in elevated adrenal cholesteryl ester concentrations. Again the differences noted in this study were smaller than those reported elsewhere (1,9).

Subjecting rats to cold stress resulted in a decrease in adrenal cholesteryl ester content. In animals receiving corn oil this amounted to about 30% of the control value and was slightly lower in the deficient rats (26%). In animals subjected to unilateral adrenalectomy (17) the ester content of the remaining adrenal 3 hr after the operation was about 40% of the value obtained for the adrenal removed in the operation.

In previous studies, subjecting rats to stress resulted in decreases in specific cholesteryl esters. Muraoka (8) found that only cholesteryl arachidonate decreased significantly in rats receiving an injection of ACTH. This conclusion was based on relative changes in the mole per cent of the esters and absolute data were not presented. Gidez and Feller (17) found that the stress of unilateral adrenalectomy performed on rats fed a commercial diet resulted in selective decreases in cholesteryl oleate, linoleate and arachidonate. They proposed the "selectivity index" employed in the present study as a means of assessing the relative utilization of each ester in relation to its concentration in the control animals. An SI in excess of unity indicates that the ester is being depleted at a rate greater than might be expected from its initial concentration in the tissue. For SI<1.0, the reverse is true. In the present study cholesteryl oleate, linoleate and arachidonate exhibited selective decreases when animals fed corn oil were subjected to cold stress. However only the last two acids were significantly lower in the stressed animals. In addition palmitoleic appeared to be selectively utilized although we were unable to demonstrate a significant decrease in the concentration of this ester. As was noted by Muraoka (8) and Gidez and Feller (17), 22:4(*n*-6), although a major constituent of this tissue, did not show any selective decrease in concentration in the stressed animals.

A slightly different pattern was noted in animals maintained on hydrogenated coconut oil and subjected to stress. The 20:3(*n*-9), 20:4(*n*-6) and 22:4(*n*-6) esters of cholesterol were significantly lower in the adrenals of the stressed animals and the latter two were very well utilized in relation to their concentrations

in the tissue, with selectivity indices of 2.8 and 2.9, respectively. They were however relatively minor constituents in the deficient tissue. On the basis of these data, the 22:4(*n*-6) ester may only be well utilized when the concentration of 20:4(*n*-6) is quite low. Both the 20:3(*n*-9) and 22:3(*n*-9) esters were selectively utilized although the decrease in the latter was not significant at the 5% level. In view of this, it seems unlikely that the reduced capacity of the adrenals from EFA deficient animals to produce glucocorticoids (13-14) can be ascribed solely to the substitution of the(*n*-9)-polyunsaturated esters for the (*n*-6)-esters. The suggestion of Hayashida and Portman (13) that the reduced steroidogenesis in EFA deficient rats stems from impairment of the energy yielding processes in the tissue must still be considered a distinct possibility.

Inclusion of erucic acid in the diet resulted in the deposition of large amounts of this acid in the adrenal cholesteryl esters as first noted by Carroll (1). The increase in plasma corticosterone in response to cold stress was smaller in animals maintained on such a diet than in those receiving olive oil. Moreover it is quite apparent from Table VI that cholesteryl erucate was poorly utilized in response to stress. Since this ester was the major constituent of the adrenals from animals in this dietary group, its lack of utilization may well account for the relatively poor response of these animals to cold stress as exemplified by the low plasma corticosterone level and the relatively small decrease in total adrenal cholesteryl esters. It is interesting to note that Beare et al. (21) found that rats receiving rapeseed oil and maintained at 4 C, lost weight and died in 4-34 days. Their mean survival time was 15 days whereas that of control animals receiving corn oil was 20 days.

Linoleate and arachidonate again exhibited selectivity indices greater than unity in animals fed the olive oil or corn-erucic diet. The SI of cholesteryl oleate in the olive oil group was only 0.8 in spite of the fact that it was the major constituent of the adrenals and was well utilized in the erucic acid group. Cholesteryl myristate was a relatively minor component of the esters from both groups and its SI was greater than unity. Again the differences between the control and deficient animals were not significant.

It appears from the data presented in this paper that the relative changes in the concentrations of specific cholesteryl esters in the rat adrenal, which may reflect the rates of hydrolysis of the esters with release of cholesterol for steroidogenesis, are dependent on the nature of the dietary fat. Cholesteryl arachidonate was consistently well utilized. Indeed, with the exception of the EFA deficient adrenals which contained very little arachidonate, the SI of this ester was remarkably constant. Cholesteryl linoleate was generally well utilized when the animals were subjected to cold stress but variable results were obtained for oleate. The unusually high concentration of cholesteryl adrenate, 22:4(*n*-6), in rat adrenal lipids does not appear to reflect a specific role for this ester as a source of cholesterol for steroidogenesis. Its only significant utilization occurred in the EFA deficient rats when the concentrations of 20:4(*n*-6) and 22:4(*n*-6) were quite low. Cholesteryl erucate was not well utilized by rats subjected to stress but it is not possible to ascribe the low plasma corticosterone level purely to the poor utilization of this acid on the basis of the current data. Other factors may be operative. For example Houtsmuller et al. (22) reported a decreased rate of synthesis of ATP in heart mitochondria from rats receiving dietary erucic acid. A similar situation may exist in the adrenal.

One of the main problems encountered in the present study was the variability of the results. The unilateral adrenalectomy technique of Gidez and Feller (17) provides a possible remedy to this problem since each animal acts as its own control. Alternatively, in vitro indubations of adrenal tissue in the absence or presence of ACTH would provide a solution to this problem. Many questions remain unanswered. The fate of the fatty acids liberated from the cholesteryl esters has not been investigated. Whether they act as sources of energy or are incorporated into other adrenal lipids is not known. Further information is required concerning the specificity of the hydrolytic enzymes in the adrenal for different cholesteryl esters. Such investigations would provide a definitive indication of the preferred substrates rather than the relative information provided by the type of experiment currently undertaken.

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A Comparison of Triglyceride, Monoglyceride, and Phospholipid Substrates for Post-Heparin Lipolytic Activities From Normal and Hypertriglyceridemic Subjects[1]

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ABSTRACT

Evidence for heterogeneity of post-heparin lipolytic activities in plasma was explored. After low or high doses of heparin, activities were measured in assays with phospholipid or monoglyceride substrate and with triglyceride substrate in assays using either calcium or albumin as the fatty acid acceptor. An excellent correlation between all assays was obtained in normals after both low and high doses of heparin over a wide range of individual response. In selected subjects with hypertriglyceridemia known to be deficient in response to heparin, all responses measured with phospholipid or triglyceride were below the lower limit of normal response. Some responses with monoglyceride substrate were within the normal range, pathophysiological evidence of a separate monoglyceridase. There was no evidence in either group of separate triglyceride lipase and phospholipase activities. The closest correlation between assays in normals was between triglyceride substrate with calcium as the fatty acid acceptor and the assay with monoglyceride. No differential effect upon lipolytic activities was found (1) after preincubation of the plasma, (2) with inhibitors in the assay, or (3) with preincubation of plasma and inhibitors prior to the assay.

INTRODUCTION

Previous studies have shown that post-heparin plasma contains, in addition to triglyceride lipase, phospholipase activity effecting the removal of the fatty acid (FA) at the α (C-1) position of phosphatidyl ethanolamine (PE) or phosphatidyl choline (1,2,3). Evidence for a parallel in vivo action of lipase and phospholipase activity, mainly on plasma very low density lipoproteins, was obtained (4). In support of an apparent identity of post-heparin lipase and phospholipase, activities with PE or triglyceride (TG) substrates appeared and dis-

[1]One of three papers being published from the Symposium, "Cholesteryl Esters," AOCS Meeting, Houston, May 1971.

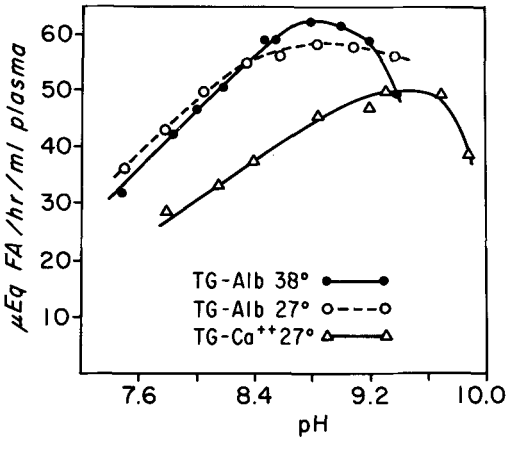

FIG. 1. Effect of pH on triglyceride hydrolysis with albumin or calcium as fatty acid acceptor. The post-heparin lipolytic activity was measured in 60 min incubations of 0.3 ml post-heparin plasma (10,000 units IV), at the temperatures and with variations of pH indicated.

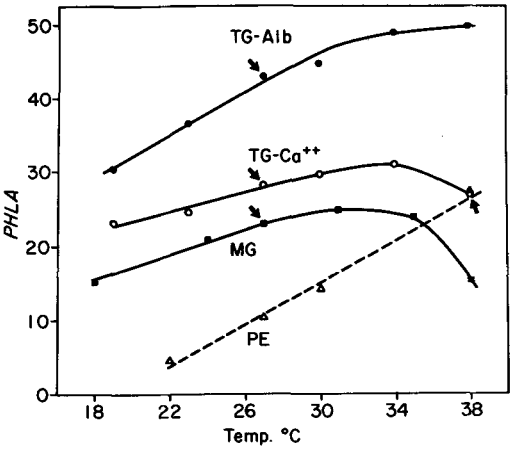

FIG. 2. The effect of temperature of incubation upon the post-heparin enzymatic activity measured in four assays. Post-heparin lipolytic activity (μ moles lyphosphatidyl ethanolamine formed, or μeq fatty acid released, per hr/ml plasma) was measured at the indicated temperatures. The arrows indicate the incubation temperature routinely used for each assay.

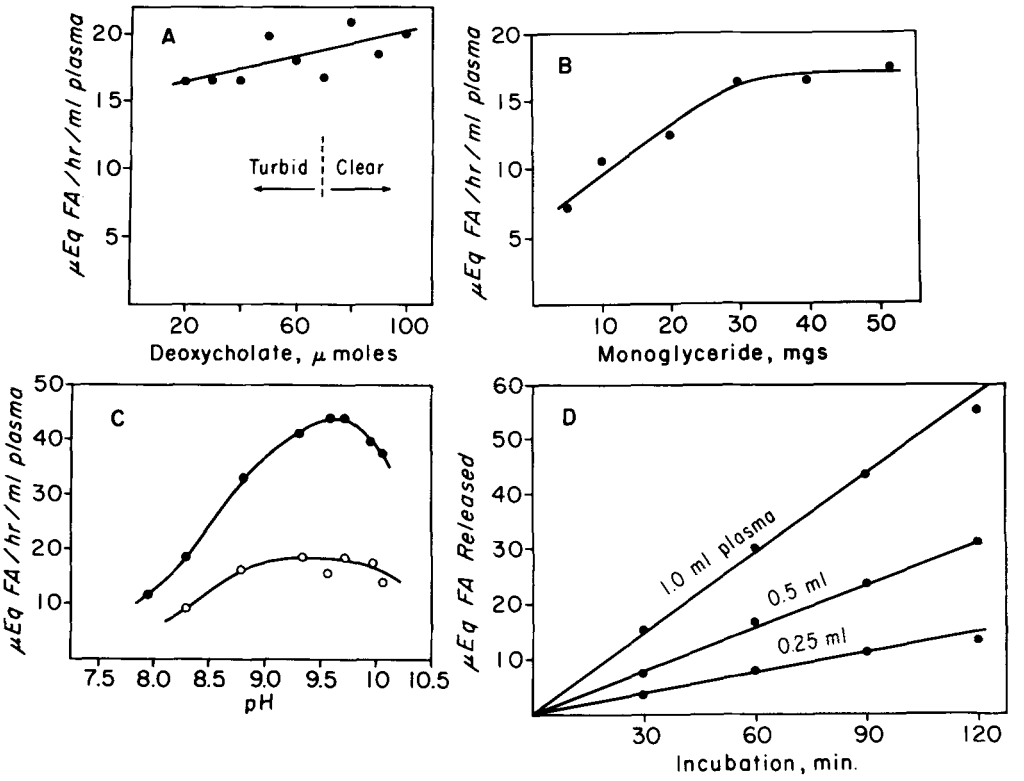

FIG. 3. The effects of the amounts of deoxycholate, substrate, and of the pH in the assay of post-heparin enzymatic activity with monoglyceride substrate. Four separate post-heparin plasmas (1,2,3,4), obtained after 10,000 units IV heparin, were used in the assay with monoglyceride substrate. In part A the amount of deoxycholate was varied (20-100 μ moles) with plasma No. 1 (0.5 ml); in part B the amount of monoglyceride was varied (5-50 mgs) with plasma No. 1 (1.0 ml); in part C the effect of pH (of complete assay at 22 C) was determined with plasmas No. 2 and No. 3 (0.5 ml each). The post-heparin lipolytic activity of plasma No. 4 was measured in part D with the amounts of plasma and intervals of incubation shown.

appeared in parallel after intravenous (IV) heparin and were not separable by various fractionation methods (5). Recently activity of post-heparin plasma on monoglyceride (MG) substrate has been documented (6,7).

This study has been designed to determine whether there is physiological evidence for heterogeneity of post-heparin lipolytic activities (PHLA) on PE, TG and MG substrates. The PHLA responses were determined in normals after both low and high doses of IV heparin. These responses were then compared to the responses obtained in subjects with hypertriglyceridemia of diverse etiology and known, by previous assay with the method of Fredrickson et al. (8), to be deficient in PHLA after a standard dose of IV heparin. In addition the possibility of differential inhibition of activities was explored.

MATERIALS

Ediol, a 50% coconut oil emulsion containing 1.5% monostearate and 2% Tween 60 (plus 12.5% sucrose and other minor components) was obtained as Lipostrate-CB (Ediol) from Calbiochem. Los Angeles, California. Intralipid (A.B. Vitrum, Stockholm, Sweden) is a 10% soybean oil emulsion stabilized with 1.2% phospholipid. PE, obtained from a crude egg yolk preparation (3) by a chromatographic separation on a column of silicic acid (1), was stored in chloroform-methanol (1:1) at -15 C. Monoolein (technical grade, T7578, Distillation Products Industries, Rochester, N.Y.), tested by thin layer chromatography (TLC), contained about 95% of material with the R_f of monoglycerides. The only apparent impurities were about equal amounts of two components which separated with an R_f of FA and an R_f of diglycerides. Since monoglycerides are relatively insoluble in petroleum ether (9), purification was accomplished as follows: an 8 cm diameter column contained 400 gm silicic acid (Mallinckrodt, 100-200

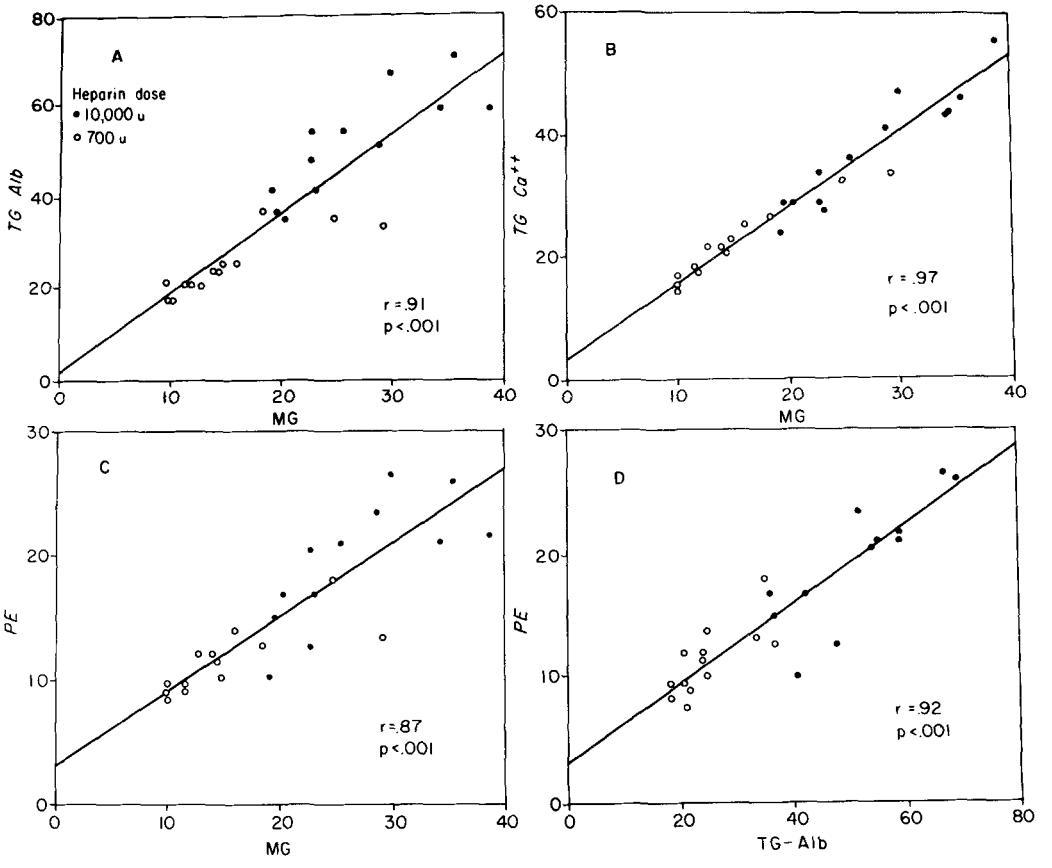

FIG. 4. Comparison of post-heparin enzymatic activities with triglyceride, monoglyceride, and phospholipid substrate in normal subjects. The solid circles and open circles designate plasmas after high and low dose IV heparin, respectively. Post-heparin lipolytic activity in the assays TG-Alb, TG-Ca++ and PE were plotted against assay MG activities in parts A, B, and C; assay PE against assay TG-Alb in part D.

mesh, activated at 125 C) in chloroform; 10 grams of crude MG was applied in 50 ml chloroform. Elution of MG was performed with 800 ml diethyl ether-chloroform (90:10 v/v) and 2000 ml diethyl ether-chloroform (70:30 v/v) in sequence. The impurities appeared in the eluates collected at 800-1100 ml; the pure monoglycerides were recovered in the eluates at 1400-2500 ml. The solvents of the latter pooled eluates were removed under partial vacuum at 45 C, the monoglyceride, tared, was stored in chloroform at 4 C. Analysis of the FA composition by gas liquid chromatography (GLC) of the pure monoglyceride showed: oleic acid 70%; 7% each of palmitoleic and stearic acids; and 16% of approximately equal amounts of five other components.

Other chemicals used include: sodium deoxycholate (No. S-285, Fisher Scientific Co., Pittsburgh, Pa.); bovine albumin, fraction V (Metrix, Armour Pharmaceutical Co., Chicago, Ill.); protamine sulfate (K and K Laboratories, Inc., Plainview, N.Y.); monoolein (602 G, highly purified, The Hormel Institute, Austin, Minn.).

METHODS

Post-Heparin Plasma Collections

Post-heparin plasma samples were obtained in the post-absorptive state from two groups of subjects. Thirteen healthy adult men ("normals") ranging in age from 28-33 years, except for one 54-year-old, were studied. Blood was collected 10 min after a standard test dose of 10 units/kg body weight and also (12 of the 13 normals) at about 20-21 min after the rapid infusion of 10,000 units of heparin (1000 units/ml, Invenex, San Francisco, California). Similarly plasma was obtained 10 min after the standard dose of heparin from 25 patients known to have hypertriglyceridemia (exogenous lipemia, untreated diabetics, hypothyroidism) and known to be deficient in PHLA response to IV heparin by the assay of

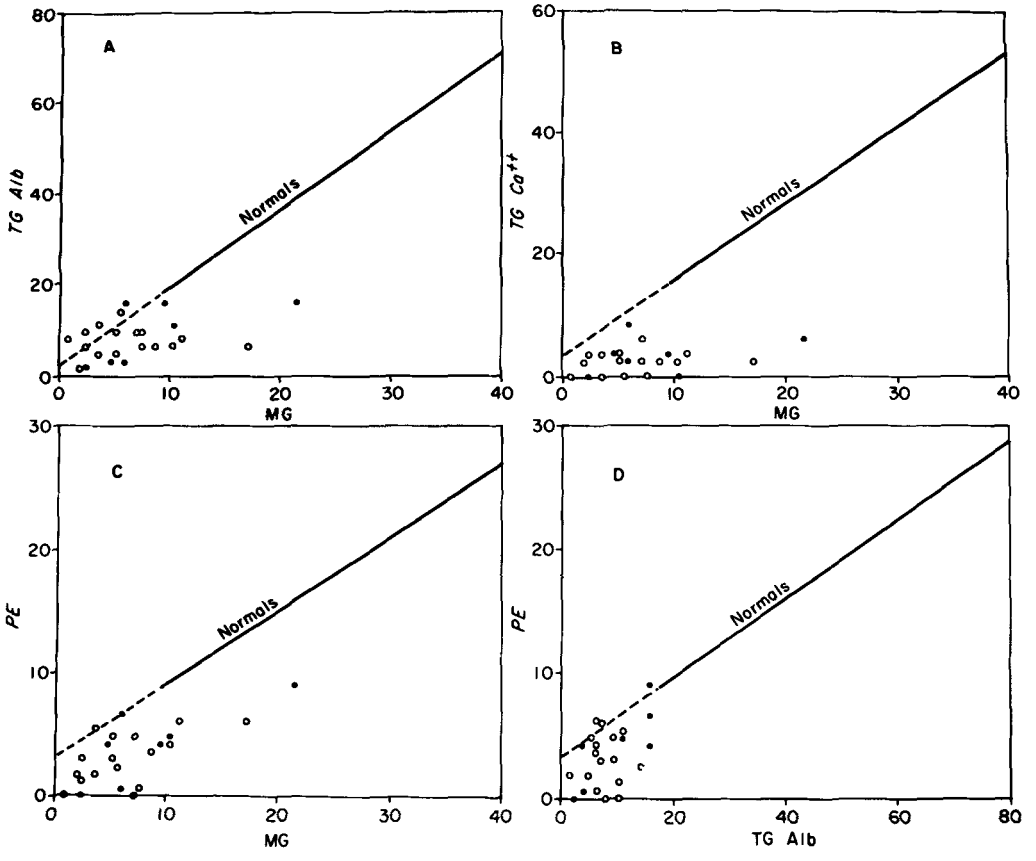

FIG. 5. Post-heparin enzymatic activities with triglyceride, monoglyceride, and phospholipid substrate in hypertriglyceridemic sujects compared to the correlations obtained from the similar assays in normal subjects. The key in Figure 4 also applies to these subjects. Regression lines in Figure 4 are plotted here but changed from a solid line to dashes at the point of lowest response obtained in normal subjects.

Fredrickson et al. (8). Plasma collections after a "high dose" of IV heparin were obtained in these subjects 60 min after an initial dose of 60 units/kg which was followed by a continuous infusion of 120 units/kg/hr (10). All blood samples were treated with anticoagulant: disodium ethylenediaminetetraacetate (1 mg/ml whole blood) or potassium oxalate (0.03 ml 33%/10 ml whole blood). The plasmas were frozen at -21 C until assayed.

Assays of Lipolytic Activity

Units of PHLA were measured as μeq FA released (with TG and MG substrate) or as μ moles lysophosphatidyl ethanolamine produced (PE substrate) per hr/ml plasma. The method of Dole and Meinertz (11) was used to measure FA released except that the concentration of H_2SO_4 in the extraction medium was doubled to compensate for the alkalinity of PHLA assays. A 1.0 ml aliquot of assay medium was taken immediately after the addition of the plasma, added to the extraction mixture, and the procedure repeated at the incubation interval chosen. A one phase titration of FA was accomplished by preparation of the indicator in absolute ethanol. Corrections of results for distribution coefficient differences for short chain FA (as in Ediol) were not made. With PE substrate, small aliquots (25-50 μl) of the assay medium taken immediately after plasma addition and at the end of the incubation interval were applied directly, without lipid extraction, to commercial silica gel loaded filter paper (12). The separation of lipids was accomplished with diisobutyl ketone-acetic acid-water (40:25:5 v/v) at 4 C for 16 hr. A spray of 0.15% ninhydrin in lutidine-acetone (10:90 v/v) was used to visualize the hydrolysis of PE. Phosphorus content of the lysophosphatidyl ethanolamine was measured as previously described (1).

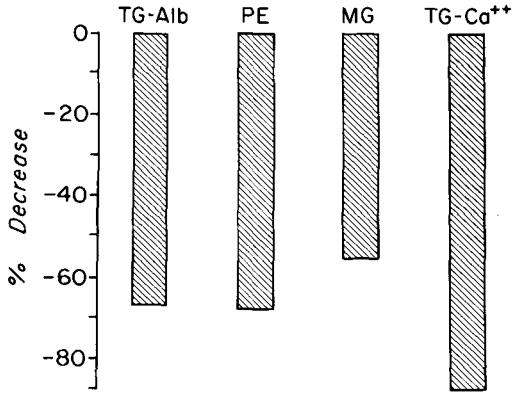

FIG. 6. The mean per cent decrease from the normal response in subjects deficient in post-heparin lipolytic activity (PHLA). The decreases in PHLA were derived from the data of Figures 4 and 5.

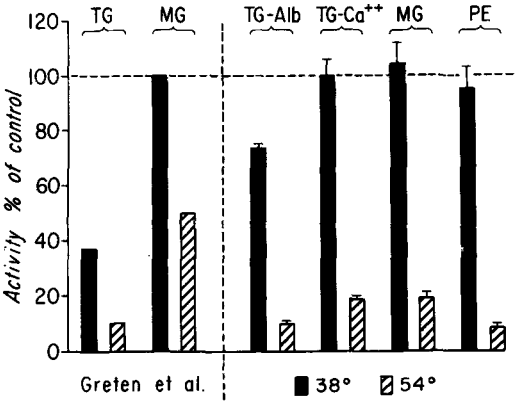

FIG. 7. The effect of preincubation of post-heparin plasma upon post-heparin enzymatic activities. The means of activities (per cent of control) with the range were plotted on the right of the vertical dotted line for each of the four assays. The results ("mean of 3 experiments") taken from Greten et al. (7,13) with triolein and monoolein substrate were plotted on the left. Solid bar-38 C, hatched bar-54 C.

Assays of PHLA

The Ediol and Intralipid were usually diluted with 0.15 M NaCl to a 5% TG content for additions to PHLA assays. Organic solvents were removed from appropriate aliquots of MG or of PE under reduced pressure at 55-60 C just prior to emulsification in the substrate medium using a tube mixer.

The four assays used are abbreviated (TG-Alb, TG-Ca^{++}, MG and PE) to indicate the substrate and in addition, with TG, to indicate the FA acceptor. All assays employed 1.0 ml post-heparin plasma (0.5 ml plus 0.5 ml 0.15 M NaCl in assays after high IV heparin with "Normals") in a total volume of 7.0 ml. Routinely a proportional reduction of components to 3.5 ml was used.

TG-Alb: incubations were for 60 min at 27 C with 1.0 ml of 50 mg Ediol in 0.15 M NaCl and 5.0 ml of solution containing 400 mg albumin and 0.45 ml 1 M $(NH_4)_2SO_4$ at pH 9.1 (with NaOH). The latter reagent was found to provide optimal activity with PE substrate (1). This assay is not appreciably different from that of Fredrickson et al. with TG substrate (8).

TG-Ca^{++}: incubations were for 60 min at 27 C with 1.0 ml of 50 mg Ediol in 0.15 M NaCl and 5.0 ml of 0.1 M glycine-0.08 M $CaCl_2$ at pH 9.6 (with NaOH). The concentration of $CaCl_2$ in the glycine buffer was set at 0.08 M since equal values of PHLA were obtained in assays over the range of 0.03-0.10 M $CaCl_2$. Excessive use of a tube mixer at the end of an incubation was avoided since calcium soaps may aggregate and deposit above the medium. (A coagulation of the assay arising from recalcification of oxalated post-heparin plasma was encountered only under the conditions of lower pH, 8.6, and lower $CaCl_2$ concentration, 0.03 M.)

Higher enzyme activities were not obtained with Ediol substrate in amounts greater than 50 mg. A decrease in rate of release of FA occurred after about 15% hydrolysis of substrate: 35 μeq FA released. Hence plasmas were reassayed using 0.5 ml when the PHLA exceeded 35 μeq FA released per hour with 1.0 ml plasma.

The PHLA of 19 plasmas correlated well with Ediol or Intralipid as substrate in the TG-Alb assay, but the mean of PHLA with Intralipid was 42% of the mean with Ediol substrate. However in the TG-Ca^{++} assay the activities with Intralipid with two plasmas were disproportionately lower, 15 and 22% of activities with Ediol substrate.

The optimal pH for measurement of PHLA in the TG-Alb assay showed a somewhat broader range at an incubation temperature of 27 C than at 38 C with less sensitivity to higher pH (Fig. 1). The optimal pH for measurement in the TG-Ca^{++} assay was appreciably higher than with the TG-Alb assay. Zero order kinetics were obtained in the TG-Alb assay at both 27 and 38 C, while with the TG-Ca^{++} assay obtained at 27 C, but not at 38 C. From studies of effect of incubation temperature over the range of 18-38 C upon assays of PHLA (Fig. 2), 27 C was selected for both TG assays.

MG: incubations were for 60 min at 27 C with 50 mg monoglyceride emulsified in 5.0 ml of 0.1 M glycine-0.02 M sodium deoxycholate

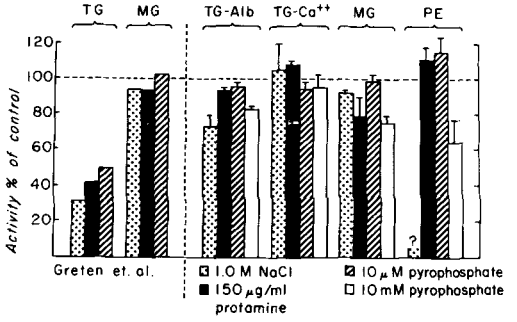

FIG. 8. The effects of inhibitors in the assays upon measures of post-heparin enzymatic activities. Comparisons as in the legend of Figure 7. The experiments with 1.0 M NaCl in the assay with phosphatidyl ethanolamine substrate were not continued since an immediate aggregation of substrate occurred.

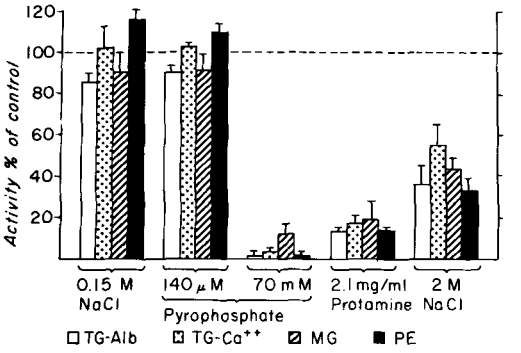

FIG. 9. The effects of the preincubation of post-heparin plasma with inhibitors upon enzyme activities. The reagent concentrations of the figure are prior to mixture (1:1) with plasma and preincubation.

at pH 9.7 (with NaOH), and 1.0 ml of 0.15 M NaCl. Chloroform must be evaporated completely from MG substrate since incomplete removal could result in cloudiness of this medium and erroneous, low, measures of PHLA.

The amount of deoxycholate in the MG assay was not critical (Fig. 3, part A), and a clear substrate was obtained at the larger amounts used. The amount of MG substrate for optimal activity in the assay was 30 mg or more (part B). An optimal pH of 9.6 for hydrolysis of substrate was indicated (part C). Zero order kinetics were found (part D) which extended to about 35% hydrolysis of substrate.

A comparison of assays with the MG prepared for substrate in this study with assays using highly purified monoolein (The Hormal Institute) showed little difference (activity with the highly purified monoolein substrate was 9.8% lower). There was no hydrolysis of the TG (50 mg) of Intralipid or of Ediol when added to the medium of this assay (0.4 and 4.0 units, respectively, per hr/ml plasma). Activity was measured from 18-38 C (Fig. 2); kinetics were studied only at 27 C (Fig. 3, part D).

PE: incubations were for 60 min at 38 C with 52 μmoles of PE emulsified in 1.0 ml 3.5% glycerol, and 5.0 ml of the same albumin and $(NH_4)_2SO_4$ reagent of the TG-Alb assay above. Except for the substrates the media were nearly identical. Measurement of lipolytic activity with PE substrate have been carried out only at 38 C (1,2,5) (Fig. 2).

The Inhibition of PHLA

The lipolytic activities of two plasma samples from normals after 10,000 units IV heparin were measured: (1) after an incubation of the plasmas at 38, 54 or 60 C for 60, 15 and 15 min, respectively; (2) after reagent additions to the substrate medium so that their concentration in the entire assay was either 1 M NaCl, 150 μg/ml protamine, 10 μM or 10 mM pyrophosphate; and (3) after preincubation at 38 C for 30 min of equal volumes of plasma and either 2 M NaCl, 0.15 M NaCl, 2.1 mg/ml protamine in 0.15 M NaCl, or 140 μM or 70 mM pyrophosphate in 0.15 M NaCl, following which the substrate was added.

RESULTS

Post-Heparin Enzymatic Activities in Normal Subjects

Activities measured in normal adults with TG, PE and MG substrate, when compared (Fig. 4), were closely interrelated. The correlation coefficients with the MG assay were: 0.91 for TG-Alb; 0.97 for TG-Ca^{++} and 0.87 for PE, an assay which has the most possibilities of analytical errors. A close correlation (r=0.92) was also observed when PHLA with PE or TG substrate in the same assay medium were compared.

The relationship among these assays is similar in plasmas obtained after low or high doses of heparin and over a wide range of individual post-heparin response.

Post-Heparin Enzymatic Activities in Hypertriglyceridemic Subjects with Low PHLA

Activities were also measured in a selected group of abnormal subjects characterized by hypertriglyceridemia and PHLA deficiency (TG substrate) using the method of Fredrickson et al. (8). The interrelationship among enzyme activities in these subjects was tested and compared to the enzyme relationships in normals (Fig. 5).

With the TG-Alb assay, responses were all

TABLE I

Comparison of Assays of Post-Heparin Lipolytic Activity[a]

Assay	Temperature, C	Volume, ml	Albumin, mg	pH	Buffer	Other additions	Substrate		
							Lipid	mg	μ moles
TG-Alb									
Greten et al. (13)	27	21.94[b]	6.7	8.6	20.01 ml 1.35 M Tris	Triton X-100	Triolein[c]	0.042	0.047
Fredrickson et al. (8)	37	5.0	500	8.4	Albumin	$(NH_4)_2SO_4$ 0.05 M	Ediol[d]	75	117
This study	27	7.0	400	9.1	Albumin	$(NH_4)_2SO_4$ 0.064 M	Ediol[e]	50	78.5
Boberg (14)	37	4.0	400	8.7	2.72 ml 0.1 M NH_4OH--NH_4Cl		Intralipid	27.9	31.4
TG-Ca^{++}									
Datta (15)	37	10.0		8.9	5.0 ml 0.1 M Tris	$CaCl_2$ 0.05 M	Ediol[f]	150	234
This study	27	7.0		9.6	5.0 ml 0.1 M glycine	$CaCl_2$ 0.057 M	Ediol[e]	50	78.5
MG									
Biale and Shafrir (6)	37	5.0		8.6[g]		Gum acacia	Monoglycerides		250-350
Greten et al. (7)	27	5.67[b]		8.6	3.34 ml 0.1 M Tris	Taurodeoxycholate 0.011 M[h]	Monoolein	6.9	19.3
This study	27	7.0		9.7	5.0 ml 0.1 M glycine	Deoxycholate 0.014 M	Monoglyceride	50	140

[a]Computations were made as necessary on the basis of 1.0 ml post-heparin plasma; pH values are those obtained prior to addition of plasma. Molecular weights of Intralipid (890) and Ediol (640) were taken from a study of Persson and Hood (17).

[b]Calculated for 0.15 ml plasma in the published assay.

[c]Amount of triolein calculated from 7.06 *n* moles (13) rather than from "7.06 *m* moles" as stated in the publication (7).

[d]Coconut oil emulsion, Schenlabs Pharmaceuticals, Inc.

[e]Coconut oil emulsion, Calbiochem.

[f]Coconut oil emulsion prepared by the investigator.

[g]Other than the higher pH, the authors used the assay of Fredrickson et al. (8) with different monoglycerides emulsified in gum acacia.

[h]Based on the selection of 5.0 mg as the correct amount in the expression "5.0 mg (0.9 *m* moles)" stated in the publication (7).

below normal as defined and some were nearly zero (Fig. 5, part A). However while most measures with MG substrate fell below the response of normals, some were at the lowest normal response level and a few were well within the range of normal. This wide distribution of activities with MG substrate was also observed in comparison with other assays: with the TG-Ca^{++} assay, part B, all samples were below normal and tended to be lower than with TG-Alb since many were at or near unmeasurable responses; with PE substrate, part C, again responses were all below normal, approached, and were in some cases measured as zero. In PHLA assays with TG and PE substrate in the same assay medium, part D, a comparable reduction in both activities was observed.

The mean per cent decrease in enzyme activities from the response obtained with normal subjects for each assay (Fig. 6) indicated a similar decrease with TG and PE substrate. The smallest decrease was found with MG substrate since a few responses of the abnormal subjects were in the normal range. The greatest decrease from normal was in assays with TG using Ca^{++} as the FA acceptor.

Effect of Preincubation Temperature on Post-Heparin Plasma

The preincubation of post-heparin plasma at 38 C for 60 min had no effect on PHLA in the four assays in the present study (Fig. 7). Preincubation of the plasma at 54 C for 15 min severely decreased all enzyme activities and at 60 C abolished them.

Effects of Inhibitors in the Assay

There was no differential inhibitory effect with any of the inhibitors when added to each of the media at the beginning of incubation (Fig. 8).

Preincubation of Post-Heparin Plasma with Inhibitors

Inhibitory effects were obtained when equal volumes of plasma and solutions of inhibitory reagents were preincubated at 38 C for 30 min (Fig. 9). With 70 mM pyrophosphate there was essentially complete inhibition in all assays. Strong and moderate inhibition of PHLA was found with protamine and 2 M NaCl, respectively, However with pyrophosphate 140 μM in 0.15 M NaCl) no effect was found compared to the control (effect of preincubation without inhibitor).

DISCUSSION

The assays used in this study are in general comparable to those employed by others (Table I); however several important differences are noteworthy. The recently introduced method of Greten et al. (13), in which a very small amount of radioactively labeled triolein as substrate and post-heparin plasma with or without delipidization was used, yielded enzymatic activity of about 0.1% of that measured in the widely used assay of Fredrickson et al. (8). The TG assay in the present study, in which substrate excess was present, is essentially comparable to those of Fredrickson et al. (8) and of Boberg using Intralipid (14).

The assay where calcium was used as the fatty acid acceptor with triglyceride substrate is a modification of the assay introduced by Datta (15) (Table I). Since uniform preparations of albumin are not assured (14,16), this study provided an opportunity to reasses albumin and calcium as fatty acid acceptors in the measurement of post-heparin lipolytic activities. Excellent correlation with monoglyceridase activity in normals was obtained. (Fig. 4).

Finally the assay in the present study with monoglyceride substrate differs from the previously published methods of Biale and Shafrir (6) and of Greten et al. (7) (Table I). At the present time there is no basis for a preferential use of bile salt as the emulsifying agent except that it provides an easier means of preparation of substrate. Biale and Shafrir, with a standardized method for stabilization of emulsified lipids with gum acacia, report higher enzymatic activity in normals than in this study, which in turn are somewhat higher than those reported by Greten et al. The method in the present study (Fig. 3, part B), and that of Biale and Shafrir contains adequate substrate to insure zero order kinetics. The rate limiting quantity of substrate in the assays of Greten et al. using either monoolein or triolein as substrate for post-heparin enzymatic activity could in part explain differences in results.

When the multiple assays were applied in a comparative study of 25 plasma samples, excellent correlations were obtained after low and high doses of heparin, over wide ranges of individual post-heparin response. Thus there was no physiological evidence of separate post-heparin activities specific for phospholipid, triglyceride or monoglyceride. Furthermore in unpublished data (Hazzard and Vogel), parallel activities measured with the four assays were found when testing dose response curves using one half to 24 times the standard dose of heparin.

Criticism of the use of Ediol as substrate in measures of post-heparin lipolytic activity has been advanced on the basis of its content of monostearin (and Tween), a substrate for a

possible specific enzyme in plasma acting only upon monoglyceride (6,7,13). Persson and Hood (17), testing the activity of human adipose tissue eluates, could not explain higher rates of lipolysis of Ediol than of other TG substrates by preferential hydrolysis of monoglycerides. In the present study the monostearin in 50 mg Ediol substrate would potentially contribute four units of activity. There is little evidence that this is a vital factor in measurements in normal subjects (Fig. 4). Furthermore there is no evidence at present that hydrolysis of triglyceride by post-heparin plasma does not proceed instantaneously to furnish an abundance of monoglyceride in vitro regardless of the nature of the substrate mixture.

With the selected patients with hypertriglyceridemia, a normal response was measured in some subjects in the assay with monoglyceride substrate, while other post-heparin enzymatic activities were deficient (Fig. 5), confirming observations of Greten et al. (7). However there was no evidence of a separate activity with phospholipid substrate. The near zero activity response in the assays using Ca^{++} as the FA acceptor is difficult to explain. The design of the assay may limit detection of very low levels of enzyme activity. In contrast, however, as an indicator of decrease from normal levels (Fig. 6), this assay may provide a greater sensitivity for decreased post-heparin enzymatic activity.

The complete inactivation of all activity upon incubation of plasma at 60 C for 15 min (7,18) was confirmed in this study. The effect of preincubation of plasma at 38 C for 60 min (Fig. 7), while decreasing activity in the TG-Alb assay, was not as pronounced as the effect observed with triolein substrate by Greten et al. The effect of preincubation at 54 C for 15 min upon the activity with monoolein substrate in this study did not appear to differentiate among the enzyme activities and was less than that reported by Greten et al (Fig. 7) and by Shore and Shore, 28-30% (18) and 18-35% (19) of control.

The differential effects of inhibitors in the assays with triolein or monoolein substrate observed by Greten et al. (Fig. 8) were not obtained in this study. Pyrophosphate at 10 mM, comparable to the concentrations found effective by Korn (20) and Korn and Quigley (21) for tissue enzyme had little effect. However marked inhibition of post-heparin lipolytic activities resulted after incubation of plasma with inhibitors prior to assay (Fig. 9). This, in part, confirms the observation of Datta and Wiggins (22), that substrate concentration is an important factor in the effects of inhibitors, protamine and NaCl, in the complete assay. With the small amount of triolein in the assay of Greten et al. (Fig. 8), and with the 22-fold dilution of the plasma, the results may be explained by decreasing enzymatic activity with progressive inhibition in a 60 min incubation at 27 C; in contrast to the results with monoolein substrate with relatively much larger amounts of substrate. Thus with post-heparin plasma the differential effect of preincubation of plasma, as suggested by Greten et al. (7), is a more productive approach than that of adding the inhibitors directly to the assay. The value of the use of inhibitors may rest on a precise determination of the conditions of concentration of inhibitors, plasma, and buffers, and of preincubation with plasma in further definition of separate post-heparin enzymatic activities.

Thus there is no evidence to date of separable post-heparin TG lipase and phospholipase activities. Pathophysiological evidence in the present study supports the possibility of a separate monoglyceridase activity.

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Cholesterol Esterification and Cholesteryl Ester Hydrolysis by Rabbit and Human Ovaries

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ABSTRACT

Esterification of cholesterol occurred in vitro in rabbit and human ovaries via an acyl CoA-cholesterol *O*-acyltransferase reaction. The rate of esterification was increased in early pregnancy and may be one of the mechanisms whereby the content of stored ovarian cholesteryl esters is increased during this period. The increased cholesteryl esters were primarily in the form of cholesteryl oleate. The cholesteryl ester fatty acid patterns in both rabbit and human ovaries differed from those in sera, suggesting that a significant portion of these esters may have been derived from in situ synthesis. The rate of hydrolysis of cholesteryl esters during pregnancy was also increased, and occurred at a faster in vitro rate than esterification. Of the agents tested, only soy lecithin was found to significantly enhance the rate of hydrolysis of cholesteryl oleate by ovarian homogenates.

INTRODUCTION

Enzymatic esterification of cholesterol has been demonstrated in rat (1), dog (2) and bovine (3) adrenals, human placenta (4) and recently in luteinized rat ovaries (5). Esterification in the latter is enhanced by prior prolactin administration.

Hydrolysis of cholesteryl esters occurs in adrenals (6,7) and in rat and rabbit ovaries (8-10). In rat ovaries this hydrolysis has been shown to be enhanced by prior treatment with leuteinizing hormone (LH) (8). Utilization of cholesteryl esters for steroid synthesis in adrenals and in rat testicular tumor mitochondria appears to require the enzymatic hydrolysis of these esters (6-11). Early in pregnancy there is a rapid increase in the amounts of cholesteryl esters stored in the ovary (10). The present investigation was designed to study the changes in ovarian cholesteryl ester metabolism occurring in early pregnancy in rabbits, and also the mechanisms of cholesteryl ester synthesis and hydrolysis in human ovaries.

MATERIALS AND METHODS

For esterification studies ovaries from nonpregnant or 6 day pregnant rabbits were homogenized with a ground glass mortar and pestle in 0.1 M phosphate buffer, pH 7.1. Human ovaries were obtained immediately after surgery from women of age range 35-48 and homogenized as were the rabbit ovaries. (The human ovaries were removed as palliative therapy for breast carcinomas. All were functional as determined by clinical and morphologic criteria.) Then 1.0 ml aliquots of the homogenates containing 100 mg of ovary were

TABLE I

Formation of Cholesteryl Esters From Precursors by Rabbit and Human Ovarian Homogenates[a,b]

	Cholesterol-4-^{14}C		Oleic-1-^{14}C	
	% Esterified	p Moles esterified/mg protein	% Esterified	p Moles esterified/mg protein
Rabbit				
Nonpregnant	1.6 ± 0.4	35 ± 8	5.7 ± 0.8	127 ± 19
6 day pregnant	2.9 ± 0.7	64 ± 15	11.2 ± 1.8	214 ± 32
Human	0.2 ± 0.1	5 ± 2.1	3.8 ± 1.1	95 ± 28

[a]Incubations were done for 1 hr at 37 C in 1.0 ml of medium containing 0.1 M phosphate buffer, pH 7.1, 6 μmoles ATP, 0.3 μmoles CoA, 5 μmoles $MgCl_2$, 100 mg tissue homogenate, and 0.05 μc, 10 n moles of each substrate.

[b]Figures given are means ± standard deviations of incubations from six separate specimens; p moles esterified are calculated assuming no equilibrium with endogenous substrate.

TABLE II

Fatty Acid Compositions of Rabbit Ovary and Serum Cholesteryl Esters[a]

Fatty acid	Nonpregnant		Pregnant, 6 days	
	Ovaries	Sera	Ovaries	Sera
16:0	17.5 ± 2.6	20.1 ± 2.7	15.6 ± 2.4	23.3 ± 3.4
16:1	3.0 ± 0.9	2.5 ± 0.7	2.1 ± 0.4	3.9 ± 0.4
18:0	15.7 ± 2.3	7.2 ± 1.8	7.0 ± 1.0	5.5 ± 0.6
18:1	39.2 ± 4.8	28.9 ± 2.8	54.1 ± 6.2	32.0 ± 4.2
18:2	20.8 ± 2.2	37.1 ± 4.2	13.4 ± 1.3	33.4 ± 5.3
18:3	1.6 ± 0.2	1.7 ± 0.3	0.5 ± 0.1	0.7 ± 0.2
20:4	2.2 ± 1.1	2.5 ± 0.4	3.3 ± 0.8	2.2 ± 1.0

[a]Percentages of totals. Means of six determinations ± standard deviations.

incubated with 0.1 ml cholesterol-4-^{14}C (Amersham/Searle, 0.05 μc, 10 n moles in each tube) or with 0.1 ml oleic acid-1-^{14}C (Amersham/Searle, 0.05 μc, 10 n moles in each) in acetone for 2 hr at 37 C with added cofactors as indicated in the tables. Control aliquots were heated at 90 C for 10 min prior to incubation. The incubations were stopped by addition of 5 ml of ethanol-acetone 1:1 and the lipids were extracted by homogenization with a ground glass mortar and pestle, centrifugation, and an additional extraction of the residue with 10 ml ethanol-acetone. Samples were evaporated to dryness under an N_2 stream, dissolved in chloroform and the lipid fractions separated by thin layer chromatography on microscope slides coated with Silica Gel H (Brinkmann) using petroleum ether/ethyl ether/acetic acid 80:20:1 as the developing solvent. The free cholesterol, free fatty acid and cholesteryl ester zones were scraped into liquid scintillation vials. Adequacy of separation was confirmed in each case by spraying a small residual edge of silica gel on the slide with 50% H_2SO_4, heating, and visualizing the lipid zones. Radioactivities in the fractions were determined by liquid scintillation counting in a PPO-POPOP-toluene scintillation solution in a Packard 3314 automatic refrigerated liquid scintillation spectrometer. Quenching was monitored by subsequent addition of internal standards.

For hydrolysis studies ovaries were homogenized with a ground glass homogenizer in 0.1 M tris-maleate buffer at pH 6.6. The 1.0 ml aliquots of the homogenates containing 100 mg of ovary plus 3 mg fat free albumin were incubated with 0.1 ml of cholesteryl-4-^{14}C-oleate in acetone (Amersham/Searle, 0.05 μc, 10 n moles in each tube) for 2 hr at 37 C. Control aliquots were heated at 90 C for 10 min prior to incubation. The incubations were stopped by addition of 5 ml of ethanol-acetone 1:1 and the lipids were extracted, separated by thin layer chromatography, and radioactivities determined as in the esterification experiments.

Cholesteryl esters isolated by thin layer chromatography from portions of each ovary were hydrolyzed and methylated (12) and the fatty acid composition of each fraction was determined by gas-liquid chromatography in a Barber-Colman Model 5000 Gas Chromatograph using diethylene glycol succinate on Gaschrom P, 70-80-mesh, at 185 C with an argon pressure of 20 lb. The fatty acids were identified by comparison of retention times to

TABLE III

Cholesterol Content of Rabbit and Human Ovaries and Sera

	Free cholesterol	Esterified cholesterol[a]
Rabbit		
Nonpregnancy ovaries	3.1 ± 0.8	6.0 ± 0.7
6 day pregnancy ovaries	3.2 ± 0.5	20.9 ± 1.9
Nonpregnancy sera	12 ± 3	30 ± 4
6 day pregnancy sera	15 ± 2	34 ± 5
Human		
Ovaries	1.4 ± 0.3	1.1 ± 0.5
Sera	67 ± 8	144 ± 18

[a]Ovarian cholesterol is given in mg/gm wet tissue, serum cholesterol in mg/100 ml sera. Both are means of determinations ± standard deviations.

TABLE IV

Fatty Acid Compositions of Human Ovarian and Serum Cholesteryl Esters[a]

Fatty acid	Ovaries	Sera
16:0	14.7 ± 2.5	13.0 ± 2.1
16:1	2.6 ± 1.0	3.9 ± 1.3
18:0	6.9 ± 2.4	2.6 ± 1.0
18:1	33.4 ± 5.4	26.4 ± 3.5
18:2	32.1 ± 6.2	45.3 ± 7.4
18:3	1.5 ± 1.1	1.4 ± 0.6
20:4	8.8 ± 1.8	7.1 ± 2.5

[a]Percentages of totals. Means of six determinations ± standard deviations.

TABLE V

Hydrolysis of Cholesteryl Oleate by Rabbit and Human Ovarian Homogenates[a,b]

Subject	% Hydrolysis	p Moles/mg protein
Rabbit		
Nonpregnant	8.2 ± 1.0	182 ± 25
6 day pregnant	21.5 ± 3.2	410 ± 45
+ cyclic 3'5' AMP 10^{-4} M	20.7 ± 3.5	385 ± 44
+ Na taurocholate 5 mg/ml	22.4 ± 3.8	397 ± 58
+ Soy lecithin 2 mg/ml	30.6 ± 4.5	575 ± 53
+ 2-amino-2 methyl-1-propanol 10^{-2} M	21.9 ± 2.9	394 ± 50
Human	12.3 ± 3.3	320 ± 62

[a]Incubations were done for 1 hr at 37 C in 1.0 ml of 0.1 M Tris-maleate buffer with 100 mg ovary homogenate, 3 mg fat free albumin, 0.05 μc, 10 n moles of cholesteryl-4-^{14}C oleate.

[b]Figures are means of six separate incubations ± standard deviations; p moles hydrolyzed are calculated assuming no equilibration with endogenous cholesteryl esters.

those of standards (obtained from National Institutes of Health and Calbiochem) and by graphic representation of retention times. The areas under the peaks were estimated by triangulation. This method estimated peak areas of a known standard fatty acid mixture within ±1-2% accuracy. Only the major fatty acids (16, 16:1, 18, 18:2, 18:3 and 20:4) have been included in the tabulations. Other fatty acids, identified in amounts either too small or having too long a retention time to measure accurately, were 14, 20:1, 22:4, 22:5 and 22:6 (these together accounted for 3-4% of the total fatty acids).

Free and esterified cholesterol content of ovaries and sera were determined by a modified Sperry-Webb procedure. Ovarian free fatty acids were determined colorimetrically by the method of Mahedevan et al. (13). Protein content of the ovarian homogenates was determined by the method of Lowry (14) adapted for the autoanalyzer.

The probabilities (P) that apparent differences in the data were due to chance were calculated by the *t* test, and only those differences where $P<0.01$ have been considered significant.

RESULTS

The extents of esterification of cholesterol-4-^{14}C and oleic acid by rabbit and human ovaries are indicated in Table I. In the rabbit, esterification with the oleic-1-^{14}C precursor was higher than with cholesterol-4-^{14}C. The ovaries on day 6 of pregnancy esterified both substrates at a significantly higher rate than the non-pregnancy ovaries. In the human ovaries, esterification with cholesterol 4-^{14}C was extremely low, but with oleic-1-^{14}C the esterification rate was 95 p moles/mg protein/hr and was in the same approximate range as in the nonpregnancy rabbit ovaries.

Incubations done over the pH range 4.5-9.0 showed dual peaks, one at 5.5 and a slightly higher one at 7.1; all subsequent experiments were done at the latter pH. Omission of both ATP and CoA from the incubation medium resulted in esterification rates approximately 10-15% of those with these cofactors present. Reduced glutathione added in concentrations of 5 μmoles/ml had no significant effects.

The cholesteryl ester fatty acid compositions of rabbit ovaries and sera and their free and esterified cholesterol contents are indicated in Tables II and III. The pregnancy ovaries contained a significantly higher percentage of oleate than did the nonpregnancy ovaries. The absolute amount of cholesteryl oleate in the pregnancy ovaries was even higher, since the latter contained approximately three times the amount of total cholesteryl esters as compared with the nonpregnancy ovaries. The percentage of stearate was significantly lower in the pregnancy ovaries. In both the nonpregnant and pregnant states the percentages of cholesteryl ester fatty acids in the ovaries were different from the sera; the latter contained more linoleate and less oleate than the ovaries. The amounts of total cholesteryl esters and the percentages of individual fatty acids were similar in sera from nonpregnant compared with sera from pregnant rabbits. The percentages of cholesteryl ester fatty acids in human ovaries and sera are indicated in Table IV. Standard deviations were somewhat higher than in the rabbit determinations, possibly due to the different ages and diets of the humans. As in the rabbits, the serum contained a higher proportion of cholesteryl linoleate and less oleate than did the ovaries.

Amounts of free fatty acids were 0.22 ±

0.03 mg/gm in the nonpregnancy rabbit ovaries, 0.27 ± 0.05 in the pregnancy rabbit ovaries and 0.17 ± 0.06 in the human ovaries. The pool size of endogenous free fatty acids therefore was not significantly different between the nonpregnancy and pregnancy rabbit ovaries, and could not have accounted for the difference in esterification rates. The same is true for the endogenous free cholesterol pools in nonpregnancy vs. pregnancy ovaries.

In Table V are indicated the degrees of hydrolysis of cholesteryl-4-^{14}C oleate during incubations with rabbit or human ovarian homogenates. Hydrolytic activity was significantly higher in the pregnancy ovaries as compared with the nonpregnant. Addition of 2 amino-2 methyl-1-propanol, sodium taurocholate or cyclic 3'5' AMP to the homogenates of pregnancy ovaries had no significant effects, but addition of 2 mg/ml of purified soy lecithin enhanced the degree of hydrolysis.

DISCUSSION

Esterification of cholesterol in both rabbit and human ovaries at pH 7.1 appears to be catalyzed by an acyl-CoA cholesterol *O*-acyltransferase, and is similar in this respect to esterification in the adrenal microsomes (3), but different from the system in the placenta (4) which apparently is a low energy esterification not requiring these cofactors. Reduced glutathione, which was found to enhance esterification in the adrenal (1), had no effect in the present ovary experiments. Adrenals in several species have been found to have a second esterification system, located in the particulate free supernatant fraction, which does not require ATP and CoA (3). Although esterifying activity was noted in the ovaries at pH 5.5, the cofactor requirements at this pH were not investigated in these experiments.

Esterification in the ovaries appeared to proceed more rapidly with exogenous oleic acid as the substrate than with cholesterol. This difference also has been noted in rat adrenals (1) and in pigeon aortas (15) and may be due to a higher rate of penetration of the fatty acids to esterification sites, or possibly to a greater dilution of the added cholesterol by the endogenous cholesterol pool. Calculation of results assuming complete equilibration of the added cholesterol and oleic acid with endogenous pools of both does produce a higher total p moles cholesterol than oleic acid incorporated into cholesteryl esters. This is probably not a valid assumption, however, since most free cholesterol is part of the membranes of the mitochondria and endoplasmic reticulum (16) and may not all be available for esterification.

The rate of esterification was increased in early pregnancy, and may be one of the mechanisms whereby the content of stored ovarian cholesteryl esters is increased during this period. Although luteinizing hormone has been shown to inhibit in vitro esterification of fatty acids with cholesterol (16), chorionic gonadotropin and prolactin appear to increase ovarian cholesteryl ester levels (16), and perhaps the increased esterification observed in the pregnant state in these experiments is mediated by the latter hormones. Prolactin recently has been found to reverse the hypophysectomy induced decline in an ovarian sterol acyl transferase in rats (5). The increased ovarian esterification of cholesterol during pregnancy appeared to be predominantly to oleic acid as judged by the increased amounts of this cholesteryl ester. The percentages of fatty acids in the ovarian cholesteryl esters in both the nonpregnant and pregnant states differed from those of the sera, suggesting that a significant portion of the ovarian esters were derived by in situ esterification. Incorporation of serum cholesterol esters into the ovary, followed by hydrolysis of some esters more rapidly than others by the ovary, however, could also result in this different fatty acid composition in ovaries vs. serum.

Hydrolysis of cholesteryl esters by the ovary is also higher during pregnancy, this effect probably being mediated by luteinizing hormone as has been shown in previous studies (8). This hydrolysis did not appear to be mediated by 3'5' cyclic AMP and was not enhanced by addition of bile acids to the media, as is sterol ester hydrolysis in pancreas and intestine (17). Lecithin stimulated hydrolytic activity, possibly by increasing the degree of physical dispersion of the emulsified cholesteryl oleate substrate, providing greater enzyme-substrate interaction. A similar enhancement of cholesteryl ester hydrolytic activity has been observed in vitro in rat intestine preparations (18) and in vivo in aortas from cholesterol-fed rabbits (19). The choline antagonist, 2-amino-2-methyl-1-propanol which has been shown to inhibit lecithin synthesis in vitro (20) had no effect in the present system, suggesting that endogenous lecithin may not have a significant role in activating cholesteryl ester hydrolysis. As well as providing increased free cholesterol substrate for steroid hormone synthesis in the ovaries during pregnancy, this hydrolytic reaction may also function to release the fatty acid moieties of the cholesteryl esters; these fatty acids have been shown to provide the major source of respiratory energy for the luteal cells (21).

Rates of hydrolysis in the ovaries were somewhat higher than rates of esterification, and if similar differences occur in vivo may indicate that a portion of the cholesteryl esters hydrolyzed by the ovary are those that have been taken up by the ovary from the plasma or lymph.

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Effect of Growth Conditions on the Fatty Acid Composition of *Listeria Monocytogenes* and Comparison With the Fatty Acids of *Erysipelothrix* and *Corynebacterium*

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ABSTRACT

Six strains of *Listeria monocytogenes* belonging to four different serotypes all had similar fatty acid profiles when grown at 37 C, with C_{15} and C_{17} branched chain acids as major components. The proportion of 17:0 br decreased markedly as the growth temperature was lowered from 37 C to 4 C, and a reduction of 18:1 with increasing age of cultures was observed in cells harvested at different stages of the growth curve. The fatty acid composition was also affected by the nature of the culture medium. Two other genera of the family Corynebacteriaceae were analyzed for fatty acid composition. Strains of *Erysipelothrix rhusiopathiae* isolated from human, turkey, dog and pig had rather similar patterns, consisting mainly of straight chain, even-numbered fatty acids from C_{10} to C_{18}. The three species of *Corynebacterium* analyzed each had quite different fatty acid patterns. *C. poinsettiae* bore some resemblance to *L. monocytogenes* but *C. pseudodiphtheriticum* had much higher proportions of 16:0 and 18:1 and *C. equi* contained a rather complex mixture of fatty acids.

INTRODUCTION

The taxonomic value of the fatty acid composition of bacteria was first suggested by Abel et al. (1) and much of the early data on bacterial fatty acids, including studies on the effects of growth conditions, were summarized by Kates (2). More recent contributions in this area have been reviewed by Kates and Wassef (3).

The fatty acids of *Listeria monocytogenes* grown at 37 C were investigated by Carroll et al. (4) and by Raines et al. (5) and the major components were found to be C_{15} and C_{17} branched chain fatty acids. During the course of further studies in our laboratory on the monocyte producing agent (MPA) of *L. monocytogenes* (6,7), different strains of the organism belonging to four main serotypes were grown in various media at different temperatures and the effects on fatty acid composition were determined. Fatty acids of cells harvested at different stages of the growth curve were also analyzed and the fatty acid composition of *L. monocytogenes* was compared with that of related members of the family of Corynebacteriaceae. The results of these studies are described below.

MATERIALS AND METHODS

L. monocytogenes strains 1 and 42, serotype

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TABLE I

Fatty Acid Composition of Various Serotypes of *L. monocytogenes* Grown in Beaulieu's Medium at 37 C

		Percentage of fatty acid[a,b]						
Strain	Serotype	14:0	15:0 br	16:0 br	16:0	17:0 br	18:0	18:1
1	I	0.9	53.0	1.1	2.6	37.7	0.7	3.4
42	I	1.8	51.6	3.3	4.0	32.3	1.0	5.8
85	II	2.1	49.9	1.4	3.8	35.8	1.6	5.1
81	III	0.4	55.0	0.6	2.0	39.5	0.6	1.7
109	IVB	0.9	58.3	2.0	2.7	36.1	—	—
163	Not typed	1.3	50.8	1.3	2.4	38.3	0.9	4.9

[a]Some minor peaks were not included in these calculations.

[b]br, Branched chain; —, not detected.

TABLE II

Fatty Acid Composition of Cells of *L. monocytogenes* Strain 109 Grown in Different Media at Different Temperatures and at Various Stages of Growth Curve

Growth medium and temperature	Optical density[a]	Percentage of fatty acid[b]									
		13:0 br	13:0	14:0 br	14:0	15:0 br	16:0 br	16:0	17:0 br	18:0	18:1
Beaulieu's medium at 37 C	0.08	Trace	Trace	0.1	1.0	53.1	1.0	5.2	34.7	0.5	4.3
	0.155	0.2	Trace	0.2	1.1	57.0	1.9	4.2	31.5	Trace	3.8
	0.25	Trace	Trace	0.3	0.7	55.2	2.4	3.6	36.4	---	1.3
	0.36	Trace	Trace	0.4	0.6	55.3	2.2	3.2	38.3	---	---
	0.425	Trace	Trace	0.4	0.4	59.0	1.2	1.3	38.2	---	---
	0.505	Trace	Trace	Trace	0.9	58.3	2.0	2.7	36.1	---	---
Trypticase soy broth (supplemented)[c] at 37 C	0.09	0.1	0.1	0.1	0.5	52.0	2.6	3.8	36.1	1.0	3.9
	0.148	Trace	Trace	Trace	2.0	44.4	3.0	14.2	34.0	Trace	2.4
	0.485	0.3	Trace	1.0	1.1	48.4	11.7	7.4	30.0	---	Trace
Tryptose broth (Difco) at 37 C	0.095	0.1	0.1	0.4	2.5	60.4	0.8	2.9	28.0	0.1	4.2
	0.185	0.4	0.1	Trace	0.5	71.3	0.7	1.0	25.6	Trace	1.0
	0.225	0.3	0.2	---	0.6	62.6	1.1	1.3	32.9	Trace	1.1
Trypticase soy broth alone at 37 C	0.132	2.5	Trace	0.9	1.1	53.1	5.1	7.9	26.0	1.2	2.1
	0.51	0.4	Trace	1.2	1.7	50.5	8.0	9.7	28.8	---	---
	0.56	0.5	Trace	1.1	11.8	40.8	5.5	23.3	16.9	---	---
Beaulieu's medium at 10 C	0.105	0.2	Trace	0.1	2.0	64.5	0.9	6.2	18.7	0.9	6.5
	0.21	0.2	0.1	0.1	1.7	64.6	0.2	6.9	17.0	2.5	6.7
	0.33	1.2	0.2	0.3	4.6	70.8	1.0	3.6	14.9	Trace	3.3
	0.45	0.7	0.3	0.2	1.6	80.4	0.5	2.0	14.3	---	Trace
Beaulieu's medium at 4 C	0.15	---	Trace	Trace	0.9	48.0	0.9	11.5	4.5	1.8	31.7
	0.25	---	Trace	Trace	1.0	69.9	0.4	7.0	3.6	1.4	16.7
	0.35	---	Trace	Trace	0.4	55.9	0.4	12.7	7.6	3.4	19.6
	0.45	---	0.8	Trace	1.2	83.6	Trace	4.5	3.6	2.2	4.1
	0.55	---	0.7	Trace	0.9	81.0	Trace	5.6	3.0	2.7	6.2
	0.60	---	0.7	Trace	0.7	85.4	Trace	4.0	5.2	1.4	2.5
Trypticase soy broth (supplemented)[c] at 4 C	0.15	1.2	1.4	---	1.0	80.8	1.4	9.6	3.1	0.3	1.2
	0.24	0.4	0.3	2.0	1.7	82.5	3.6	1.2	7.3	0.1	0.7
	0.69	1.1	2.2	12.0	7.7	69.1	3.4	1.6	5.5	---	0.3
Tryptose broth (Difco) at 4 C	0.155	0.8	0.3	Trace	5.1	77.9	Trace	3.6	9.5	0.5	2.4
	0.255	1.0	0.7	0.1	5.4	80.6	0.1	2.5	7.9	0.3	1.3
Trypticase soy broth alone at 4 C	0.115	1.0	0.4	1.4	2.5	82.0	1.7	1.7	7.5	0.4	1.4
	0.29	1.4	2.5	5.4	2.8	77.8	2.8	1.4	4.6	Trace	0.9
	0.65	3.1	1.0	6.0	20.6	59.2	2.2	4.2	3.7	---	Trace

[a]The highest optical density corresponds in each case to the stationary phase.

[b]br, Branched chain; ---, not detected.

[c]Supplemented with dextrose, Na_2HPO_4, thiamine HCl and NaCl.

TABLE III

Fatty Acid Composition of *Erysipelothrix* and Species of *Corynebacterium* Grown at 36 C[a]

Species	Percentage of fatty acid[b]													
	10:0	12:0	14:0 br	14:0	15:0 br	15:0	16:0 br	16:0	16:1	Unkn.[c]	17:0 br	17:0	18:0	18:1	Unkn.[c]
E. rhusiopathiae															
Strain															
337 (Human)	8.3	13.0	---	4.8	1.3	0.3	---	28.3	1.5	---	---	---	18.7	23.8	---
597 (Turkey)	8.5	5.9	---	5.0	10.0	0.6	---	25.5	2.7	---	1.6	---	13.7	26.5	---
598 (Dog)	15.9	10.1	---	4.3	1.4	0.3	---	22.2	2.0	---	0.3	---	14.9	28.6	---
599 (Pig)	20.2	11.6	---	3.4	10.6	0.5	---	17.7	1.6	---	1.5	---	10.0	22.6	---
C. poinsettiae	---	---	0.7	0.5	33.9	---	18.1	3.7	---	---	40.5	---	1.0	1.6	---
C. pseudodiphtheriticum	---	---	0.2	1.1	26.2	0.9	1.4	21.8	2.6	---	5.0	---	2.6	38.2	---
C. equi	---	---	1.9	8.5	24.0	3.4	3.0	30.5	1.9	2.7	7.9	1.1	3.4	4.0	7.7

[a]*Erysipelothrix* grown in Beaulieu's medium and *Corynebacterium* grown in yeast extract.

[b]br, Branched chain; ---, not detected.

[c]Unkn., not identified. These fatty acids coincided with 16:1 and 18:1 on the polar column (Fig. 1) and had the same retention times relative to 16:0 and 18:0 respectively, on both polar and non-polar columns.

1; 81, serotype 3; 85, serotype 2; 109, serotype 4B; and 163, unknown serotype, were studied. Strain 1, NCTC 357 and ATCC 15315, was isolated by Murray et al. (8). Strain 42 was originally isolated by Stanley (9) in Australia from a case of human meningitis and was used in the early studies on MPA. Strains 81 and 85, Paterson strains NCTC 5105 and 5348 respectively, and strain 109, Universität Würzburg 1071/53, were obtained from Professor H. Seeliger, Institut für Hygien iind Microbiologie der Universität Würzburg; strain 163 was isolated in 1965 from human cerebrospinal fluid at McGill University, McGill University 32872. Three species of *Corynebacterium* obtained from McGill University, *C. equi* 338, *C. poinsetiae* 254 and *C. pseudodiphtheriticum*, together with four strains of *Erysipelothrix rhusiopathiae*, University of Western Ontario 337 isolated from human, 597 isolated from turkeys, 598 isolated from a case of canine endocarditis and 599 isolated from pigs, were also studied. The bacteria were kept in the lyophilized state and during experiments strains of *L. monocytogenes* were maintained on tryptose agar, Difco, and those of *Corynebacterium* and *Erysipelothrix* were maintained in Brewer's meat or on blood agar, or both, at 4 C respectively.

Media and Conditions of Growth

The following media were used for the growth of *L. monocytogenes*: (a) Beaulieu's medium (10) which is composed of 2.7% tryptose broth (Difco), 0.2% dextrose, 0.319% Na_2HPO_4, 0.0005% thiamine HCl and 0.5% NaCl; (b) 2% tryptose broth (Difco) alone; (c) trypticase soy broth (BBL) supplemented with 2% dextrose, 0.319% Na_2HPO_4, 0.0005% thiamine HCl and 0.5% NaCl; and (d) trypticase soy broth alone. Beaulieu's medium, and broth containing 0.2% yeast extract and 0.1% glucose at pH 7.0, were used for the growth of cells of *Erysipelothrix* and *Corynebacterium* respectively.

Cultivation of the microorganisms in the fluid media was carried out in Erlenmeyer flasks of 0.5 and 2 liters capacity, containing 250 or 1000 ml of the broth respectively. The cultures were shaken at a rate of 105-128 excursions per min at 37 C for 8-24 hr, at room temperature for 24-48 hr or at 4 C for three to eight days. The cells were checked for purity and harvested live either at various stages of their growth phase or at their stationary phase. They were then washed and lyophilized as reported previously (6).

Fatty Acid Analysis

Lipids were extracted as described previ-

ously (6) and methyl esters of the fatty acids were prepared from the crude lipid extracts by refluxing for 2 hr with HCl-methanol prepared by adding 10% (v/v) of acetyl chloride dropwise to methanol (4). In some experiments the methyl esters were prepared by refluxing the lyophilized cells directly for several hours with the transmethylating reagent. The cooled mixture was filtered and the methyl esters were then extracted with Skellysolve B. The samples were either analyzed immediately or after storage at -20 C.

The fatty acid esters were analyzed by gas liquid chromatography (GLC) on a 6 ft x 1/8 in. nonpolar column consisting of 3% SE-30 on siliconized Chromosorb W in a Barber-Colman Model 5340 with thermal conductivity detector and on a 10 ft x 1/8 in. polar column of 15% EGSS-X on Chromosorb P in a Beckman GC-45 with hydrogen flame detector. The columns were operated at 180 C and 200 C respectively. Identification of the fatty acids was based on comparison with the retention times of methyl ester standards (NIH) and also by comparative chromatography of hydrogenated or brominated samples (11) to distinguish between saturated and unsaturated fatty acids. The product of peak height multiplied by retention time was used for quantitation (12) and the results were checked by comparison with NIH standard mixtures.

RESULTS

The fatty acid composition of six different strains of *L. monocytogenes* grown at 37 C is shown in Table I. The results were basically similar to those obtained previously (4,5) and the differences between strains were no greater than those observed when a particular strain was harvested at different stages of the growth curve (Table II). From this Table it can be seen that the fatty acid pattern is also affected by the growth temperature and the growth medium. In particular the proportion of 17:0 br was much lower at 4 C than at higher temperatures. The low proportion of 17:0 br at 4 C was observed with all six of the strains listed in Table I. In most cases the difference was made up mainly by 15:0 br but in Beaulieu's medium there was a high proportion of 18:1 during the early stages of the growth curve and in trypticase soy broth the proportion of C_{14} increased at the end. In addition Table II shows that the yield of cells as measured by optical density was also affected by the growth medium. Trypticase soy appeared to be a more suitable medium for *L. monocytogenes*, since without addition of the four supplements it gave yields comparable to those with Beaulieu's medium.

Four different strains of *Erysipelothrix rhusiopathiae* obtained from various sources were grown at 37 C under conditions comparable to those of the *L. monocytogenes* described in Table I. Results of the fatty acid analysis are shown in Table III. The different strains contained similar mixtures of fatty acids with 16:0, 18:0 and 18:1 as major components. The fatty acid profiles of *E. rhusiopathiae* were quite different from those of *L. monocytogenes* and an interesting feature was the presence of substantial amounts of two medium chain fatty acids, 10:0 and 12:0. The strains isolated from turkey and pig contained about 10% of branched chain C_{15} fatty acid but strains from human and dog had only 1-2%.

The fatty acid patterns of three strains of *Corynebacterium* grown in yeast extract at 37 C under aerobic conditions are also shown in Table III. It was not possible to grow these in either Beaulieu's medium or the trypticase soy medium. These organisms each contained three or four major fatty acids with smaller proportions of a number of others, but the patterns differed quite markedly in the different strains. *C. poinsettiae* resembled *L. monocytogenes* in its high content of 15:0 br and 17:0 br fatty acids, but contained more 16:0 br. *C. pseudodiphtheriticum* also had a considerable amount of 15:0 br but very little 17:0 br, and 18:1 was much more prominent than in *C. poinsettiae*. *C. equi* contained a more complex mixture of fatty acids with 15:0 br and 16:0 in largest amounts.

In some cases the fatty acid methyl esters were reanalyzed by GLC on a 10 ft polar column which gave partial separation of the iso and anteiso forms of the odd number fatty acids. It can be seen in Figure 1 that anteiso acids predominated in *L. monocytogenes, E. rhusiopathiae, C. poinsettiae* and *C. equi* whereas *C. pseudodiphtheriticum* contained mainly the iso form. These results were obtained with extracts from bacteria grown to the stationary phase. However with *L. monocytogenes* similar proportions of iso to anteiso forms were observed with cells harvested at earlier stages of the growth cycle and the use of either Beaulieu's medium or trypticase soy broth did not seem to have any marked effect on the ratio of the isomers.

DISCUSSION

The analytical data obtained in this study show that the fatty acid composition of *L. monocytogenes* is affected by the conditions under which it is grown. The major fatty acids

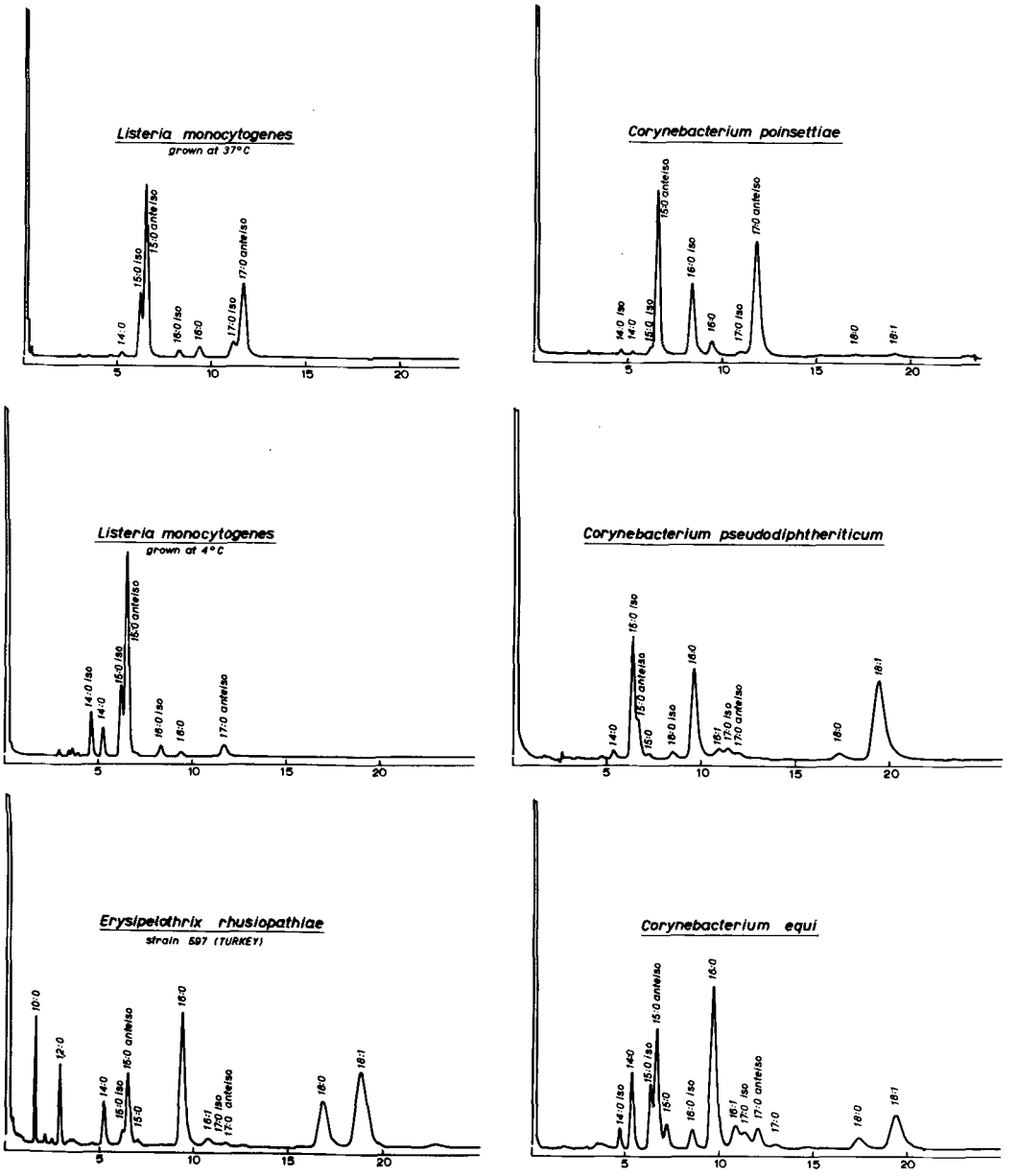

FIG. 1. GLC of bacterial fatty acid methyl esters run on a 10 ft x 1/8 in. column packed with 15% EGSS-X (Applied Science Laboratories, State College, Pa.) on acid-washed Chromosorb P (100/120 mesh, Supelco, Inc., Bellefonte, Pa.) Carrier gas: He, 60 ml/min. Injector 250 C, column temperature 200 C, hydrogen flame detector 250 C.

are 15:0 br and 17:0 br in cells grown at 37 C but the amount of 17:0 br decreases markedly as the environmental temperature is lowered. Other fatty acids such as 14:0, 14:0 br, 16:0 and 18:1 are sometimes present in appreciable amounts depending on the temperature, growth medium and the age of the culture (Table II).

There did not seem to be any great differences in fatty acid composition between different strains of *L. monocytogenes* grown under the same conditions, indicating that fatty acid profiles are of little value for typing strains in this genus (Table I). This confirms the work of Raines et al. (5). On the other hand comparison of the fatty acids of *Listeria* with those of *Erysipelothrix* and *Corynebacterium* shows that there are fundamental differences in their fatty acid patterns which are useful for differenti-

ation and identification of these three genera in the family of Corynebacteriaceae (Fig. 1).

We are not aware of previous studies on the fatty acid composition of *Erysipelothrix* but fatty acid analyses have been carried out on a number of different species of *Corynebacterium.* Early studies dealing mainly with *C. diphtheriae* were reviewed by Asselineau (13) and more recently Moss et al. (14) have studied the fatty acid composition of several anaerobic species, particularly *C. acnes.* Our results showed substantial differences among the fatty acid patterns of three species of *Corynebacterium* which were on the whole greater than the differences observed by Moss et al. in their studies of six different species grown under anaerobic conditions. It may be noted however that Barksdale (15) has recently suggested that *C. poinsettiae,* one of the species used in our studies, should be dropped from the Genus *Corynebacterium* because it differs too much from the description of the type species. He also recommended that *C. acnes* be removed from this Genus for similar reasons.

The results of our fatty acid analysis of *C. pseudodiphtheriticum* were somewhat different from those reported for *C. hofmanii* by Welby-Gieusse et al. (16), although the two names appear to be synonymous (17,18). *C. hofmanii,* like *C. diphtheriae* and some other species of *Corynebacterium,* also contains complex fatty acids of the corynomycolic type (13,16,19), but these were not investigated in our studies.

The present results provide further evidence that analysis of fatty acid composition can be a valuable aid in classification of bacteria, but caution must be used in interpreting the findings. The results obtained with *Listeria* emphasize the importance of making comparisons under well-controlled and comparable conditions of growth in order to avoid misleading results.

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Distribution of Free and Conjugated Sterols in Orange and Tangor Juice Sacs

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ABSTRACT

Comparative studies of the sterol composition of four sterol fractions, vis., free sterols, sterol esters, sterol glucosides and esterified sterol glucosides, were conducted on the juice sacs of six varieties of oranges and two tangor varieties. The sterols quantified in each fraction were β-sitosterol, campesterol, stigmasterol, cholesterol, 24-ethylidene cholesterol, brassicasterol and 24-methylene cholesterol. Each variety showed its own intrinsic composition for these sterols in the four sterol fractions.

INTRODUCTION

Extensive reviews on the biosynthesis of sterols in higher plants (1-4) indicate that sterols are formed through a complicated series of reactions. Cyclization of squalene produces tetracyclic triterpene alcohols which are metabolized to 4α-methyl steroid compounds which in turn are demethylated to form the sterol group. By strict definition (5) a sterol contains only two methyl groups attached to the perhydro-1,2-cyclopentanophenanthrene ring at positions 10 and 13. This definition excludes the triterpene alcohols and 4α-methyl steroid compounds which have often been loosely classed as 4,4-dimethyl and 4α-monomethyl sterols respectively. This paper defines a sterol according to its strict definition.

Studies on sterols in citrus fruits have been very limited. Sterols have been reported in the rinds (6), pulp (7) and juice (8) of Valencia oranges but definitive sterol characterizations were not undertaken. Of all citrus, the peel of grapefruit has been subjected to the most intensive sterol investigations. By a combination of gas chromatography-mass spectra and other methods, stigmasterol, β-sitosterol, campesterol, cholesterol and 24-ethylidene cholesterol were definitely identified in grapefruit peel (9-12). To date, the $\Delta^{5,7,22}$ sterol, ergosterol, has been shown to occur in only one citrus, i.e., peel of Rangpur lime (13).

Sterols are distributed in citrus (14,15) and other higher plants in four fractions, viz., free sterols (FS), sterol esters (SE), sterol glucosides (SG) and esterified sterol glucosides (ESG). To the authors' knowledge, studies on the comparative distribution of sterols in each of these four fractions have never been undertaken. The purpose of the present study was to determine the comparative distribution of the most prominent sterols in citrus juice sacs, viz., Δ^5, $\Delta^{5,22}$ and $\Delta^{5,24(28)}$ sterols, in each sterol fraction. Sterols were studied in six orange varieties of *Citrus sinensis* Osbeck, viz., Walker Early, Parson Brown, Hamlin, Washington Navel, Pineapple and Valencia, and two tangor varieties, viz., Temple (*C. sinensis* x *C. reticulata*) and Temple x Kinnow [(*C. sinensis* x *C. reticulata*) x *C. reticulata*].

MATERIALS AND METHODS

Isolation and Purification of Juice Sac Lipids

Valencia, Hamlin, Parson Brown and Walker Early oranges were obtained from local groves. Pineapple and Washington Navel oranges and the Temple x Kinnow tangor were obtained from Whitmore Experimental Farm (Crops Research Division, USDA, Orlando, Fla.). The Temple tangor was obtained from a local market. All samples were collected at the time of their respective peak maturities. The eight citrus were cut in half and the intact juice sacs (vesicles) carefully separated from core, peel, seeds and carpellary membrane with the aid of a citrus spoon. The juice sacs were freeze-dried to a powder possessing a moisture content no greater than 4% and stored at 5 C until lipid extractions were carried out. Lipids were extracted and purified from 15 g of juice sac powder by the method previously described for orange juice powders (14). Each variety sample was run in triplicate.

Column and Thin Layer Chromatography (TLC)

The total purified lipid (ca. 150 to 200 mg) was dissolved in absolute $CHCl_3$ (no ethanol stabilizer) and percolated onto a 0.9 x 30 cm column containing 9 g Merck, 70-325 mesh silica gel (Brinkmann Instruments, Westbury, N.Y.). Neutral lipids containing the free sterol and sterol ester fractions were eluted with 150 ml absolute $CHCl_3$ and glycolipids containing the sterol glucoside and esterified sterol gluco-

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side fractions were eluted with 200 ml $CHCl_3$/MeOH (95:5). Column aliquots (25 ml) were monitored by TLC to insure elution completeness for each group. The total neutral lipid fraction was concentrated to a small volume and streaked on precoated Silica Gel G plates (20 x 20 cm, 250μ, Analtech, Inc., Wilmington, Del.). These nonactivated plates were developed at room temperature in chambers lined with filter paper in hexane-ethyl ether (90:10). The bands corresponding to the free sterol and sterol ester fractions were scraped from the plate and eluted from the gel with ethyl ether. The glycolipid fraction was streaked on precoated G plates and developed in $CHCl_3$/MeOH (85:15). This solvent separated the glycolipid fraction into cerebrosides, sterol glucosides, monogalactosyl diglycerides, esterified sterol glucosides and resin acids.

For detection of glycolipids, anisaldehyde-H_2SO_4 and α-naphthol-H_2SO_4 (16) sprays were employed; and for sterol containing lipids, spraying with 50% sulfuric acid followed by heating at 140 C for 10 min produced distinct colors. Glycolipids, separated by preparative TLC, were detected by spraying with the nondestructive Rhodamine 6 G and viewed under UV light. The SG and ESG fractions were scraped from the plate and eluted with $CHCl_3$/MeOH (1:1). Because of the weak solubility of SG in $CHCl_3$/MeOH solvents, small amounts of pyridine were often added to maximize SG solubility during transfer. Free sterols and sterols obtained from degradation of SE, SG and ESG were separated from triterpene alcohols and 4-methyl steroid compounds by development on precoated G plates in hexane-ethyl acetate 4:1.

Degradation Studies

The SE fraction was concentrated to dryness under nitrogen and hydrolyzed with 3 ml 6% KOH in 95% ethanol in 10 ml sealed acetylation tubes (Regis Chemical Co., Chicago, Ill.) for 1 hr. After neutralization the products were extracted into cyclohexane and concentrated. The hydrolyzed sterols were separated from the free fatty acids by chromatography on G plates with $CHCl_3$ as solvent. The sterol fraction was eluted, restreaked on G plates and developed in hexane-ethyl acetate 4:1.

The SG and ESG fractions were concentrated to dryness and hydrolyzed by reacting the sample in sealed acetylation tubes with 4 ml 0.5N anhydrous HCl in MeOH containing 0.1% di-tert-butyl-cresol for 22 hr at 75 C. The hydrolysis mixture was concentrated to dryness, taken up in cyclohexane, streaked on G plates and developed in hexane-ethyl acetate.

TABLE I

Distribution of Sterols in Free Sterol Fraction (%)

Sterol	Cultivar							
	W. Early	P. Brown	Hamlin	W. Navel	Pineapple	Valencia	Temple	Temple x Kinnow
Cholesterol	1.46 ±0.02[a]	1.60 ±0.12	1.53 ±0.06	1.55 ±0.05	1.57 ±0.06	1.86 ±0.17	2.91 ±0.11	0.64 ±0.09
Campesterol	13.48 ±0.15	13.49 ±0.17	13.82 ±0.12	13.61 ±0.14	12.40 ±0.11	15.97 ±0.17	12.83 ±0.12	10.86 ±0.13
Stigmasterol	5.05 ±0.17	6.06 ±0.03	5.80 ±0.09	3.86 ±0.12	4.83 ±0.14	6.87 ±0.18	5.61 ±0.12	4.31 ±0.16
Sitosterol	78.75 ±0.85	75.98 ±0.16	74.64 ±0.27	77.42 ±0.47	76.52 ±0.22	73.85 ±0.50	74.20 ±0.05	81.62 ±0.61
24-ethylidene cholesterol	1.25 ±0.39	2.88 ±0.27	4.21 ±0.20	3.55 ±0.46	4.69 ±0.02	1.45 ±0.78	4.46 ±0.11	2.58 ±0.52
24-methylene cholesterol	T[b]	T	T	T	T	T	T	T
Brassicasterol	T	T	T	T	T	T	T	T

[a]mean ±standard deviation of 3-6 determinations.

[b]trace, less than 0.01%.

TABLE II

Distribution of Sterols in Sterol Ester Fraction (%)

Sterol	Cultivar							
	W. Early	P. Brown	Hamlin	W. Navel	Pineapple	Valencia	Temple	Temple x Kinnow
Cholesterol	2.80 ± 0.22	2.01 ± 0.10	2.68 ± 0.41	2.36 ± 0.14	2.42 ± 0.49	2.29 ± 0.11	3.40 ± 0.06	3.03 ± 0.31
Campesterol	10.96 ± 0.04	9.43 ± 0.11	9.56 ± 0.07	11.04 ± 0.05	8.45 ± 0.11	13.17 ± 0.04	11.15 ± 0.06	8.87 ± 0.08
Stigmasterol	0.51 ± 0.09	0.64 ± 0.02	0.60 ± 0.01	0.38 ± 0.07	0.70 ± 0.04	1.02 ± 0.04	0.60 ± 0.04	0.49 ± 0.08
Sitosterol	83.13 ± 0.09	80.07 ± 0.13	78.90 ± 0.22	77.26 ± 0.68	79.86 ± 0.27	78.27 ± 0.27	74.77 ± 0.17	82.32 ± 0.31
24-ethylidene cholesterol	2.49 ± 0.30	7.74 ± 0.31	8.20 ± 0.24	8.63 ± 0.85	8.42 ± 0.32	5.01 ± 0.08	9.98 ± 0.31	4.94 ± 0.20
24-methylene cholesterol	0.08 ± 0.02	0.10 ± 0.01	0.05 ± 0.01	0.10 ± 0.03	0.13 ± 0.05	1.02 ± 0.05	0.07 ± 0.02	0.31 ± 0.08
Brassicasterol	0.03 ± 0.01	0.01 ± 0.01	0.04 ± 0.02	0.01 ± 0.01	0.03 ± 0.00	0.02 ± 0.01	0.01 ± 0.01	0.03 ± 0.01

TABLE III

Distribution of Sterols in Sterol Glucoside Fraction (%)

Sterol	Cultivar							
	W. Early	P. Brown	Hamlin	W. Navel	Pineapple	Valencia	Temple	Temple x Kinnow
Cholesterol	0.32 ± 0.01	0.34 ± 0.05	0.33 ± 0.04	0.41 ± 0.05	0.33 ± 0.00	0.32 ± 0.02	0.78 ± 0.05	0.22 ± 0.04
Campesterol	8.61 ± 0.16	8.71 ± 0.34	9.09 ± 0.26	8.48 ± 0.21	7.97 ± 0.05	10.42 ± 0.08	8.83 ± 0.16	7.45 ± 0.03
Stigmasterol	2.22 ± 0.06	2.22 ± 0.38	2.55 ± 0.10	2.00 ± 0.42	2.03 ± 0.02	3.37 ± 0.06	2.24 ± 0.09	1.72 ± 0.01
Sitosterol	88.49 ± 0.19	87.93 ± 0.16	86.85 ± 0.27	88.04 ± 0.36	88.47 ± 0.10	85.51 ± 0.07	87.15 ± 0.21	89.61 ± 0.07
24-ethylidene cholesterol	0.37 ± 0.04	0.80 ± 0.17	1.19 ± 0.06	1.07 ± 0.11	1.19 ± 0.06	0.38 ± 0.08	1.01 ± 0.07	1.00 ± 0.00

TABLE IV

Distribution of Sterols in Esterified Sterol Glucoside Fraction (%)

Sterol	Cultivar							
	W. Early	P. Brown	Hamlin	W. Navel	Pineapple	Valencia	Temple	Temple x Kinnow
Cholesterol	0.46 ± 0.07	0.38 ± 0.02	0.49 ± 0.06	0.32 ± 0.04	0.32 ± 0.04	0.31 ± 0.06	0.86 ± 0.09	0.23 ± 0.08
Campesterol	7.53 ± 0.21	7.77 ± 0.08	7.68 ± 0.07	7.48 ± 0.60	7.15 ± 0.01	9.13 ± 0.10	7.66 ± 0.07	6.47 ± 0.05
Stigmasterol	2.48 ± 0.18	2.89 ± 0.16	2.77 ± 0.06	2.21 ± 0.03	2.44 ± 0.02	3.53 ± 0.09	2.14 ± 0.13	2.13 ± 0.09
Sitosterol	89.44 ± 0.31	88.66 ± 0.20	88.82 ± 0.14	89.48 ± 0.54	89.71 ± 0.04	86.80 ± 0.07	88.95 ± 0.15	90.85 ± 0.14
24-ethylidene cholesterol	0.09 ± 0.05	0.31 ± 0.18	0.23 ± 0.19	0.51 ± 0.01	0.38 ± 0.07	0.26 ± 0.06	0.38 ± 0.04	0.33 ± 0.07

Gas Liquid Chromatography (GLC)

The purified sterol fraction obtained from preparative TLC was trimethylsilylated (TMS) by reacting 1-10 mg free sterol with 0.3 ml N,O-bis-(trimethylsilyl)-acetamide. The mixture was heated at 60 C for 15 min before analysis by GLC. All samples were analyzed with an F and M Model 5750 gas chromatograph equipped with hydrogen flame detectors. The sterol-TMS derivatives were separated on a 12 ft x 1/4 in. glass column packed with 4% OV-210 and 1% OV-17 coated on 100-120 mesh Gas Chrom Q (Applied Science Labs., State College, Pa.). Separations were accomplished by on-column injection employing the following temperatures: column, 230 C; injection port, 255 C; and detector, 290 C. The helium flow rate was 80 ml/min. Quantitative results were obtained by triangulation measurement techniques coupled with measurement of peak areas with the aid of a disc integrator.

Mass Spectra

Sterol-TMS samples were separated by GLC and captured in glass capillary tubes. These samples were analyzed by the direct inlet probe technique with a CEC Model 21-490 mass spectrometer (Bell and Howell, Monrovia, Calif.). The ionizing temperature was 250 C, probe temperature 180 C, ionizing voltage 70 eV and spectra recorded at scan speeds of 4 sec/decade and 10 sec/decade. Mass spectral identification of juice sac sterols has been previously determined (17).

RESULTS AND DISCUSSION

The amount of lipid extracted from 15 g of powdered juice sacs ranged from 163 mg (Pineapple) to 230 mg (Temple). These values indicate that orange and tangor juice sacs are composed of 1.1-1.5% lipid on a dry weight basis. The weight percentage distribution of the four sterol fractions has been determined only for Valencia oranges (18). From 15 g of Valencia orange juice sac powder, 185 ± 4 mg (1.2%) of purified lipid was obtained. By a combination of TLC densitometry (14) and gravimetric determinations, the following weight percentages have been computed for the four sterol fractions: free sterols, 8.1%; sterol esters, 6.2%; sterol glucosides, 8.6%; and esterified sterol glucosides, 1.7%.

In orange and tangor juice sacs the following Δ^5 sterols have definitively been characterized by mass spectra, viz., cholesterol, campesterol and β-sitosterol. The $\Delta^{5,22}$ sterols found were stigmasterol and brassicasterol and the $\Delta^{5,24(28)}$ sterols were 24-ethylidene cholesterol and 24-methylene cholesterol.

Table I shows the distribution of these seven sterols in the free sterol fraction of orange and tangor juice sacs. β-sitosterol is the most dominant sterol comprising ca. 75% of this fraction. The order of concentration for these seven sterols is β-sitosterol > campesterol > stigmasterol > 24-ethylidene cholesterol ≳ cholesterol. The two sterols, 24-methylene cholesterol and brassicasterol, are found in all varieties at trace concentrations, i.e., less than 0.01%. Except for minor differences, Walker Early, Parson Brown, Hamlin, Washington Navel and Pineapple oranges possess essentially similar distributions for these seven sterols. One noticeable departure is the low percentage of 24-ethylidene cholesterol in the Walker Early orange. As observed in Tables II, III and IV this low percentage is consistent in all sterol fractions. Valencia orange shows deviations from the other five orange varieties by the presence of higher campesterol and stigmasterol percentages. The two tangors show distinct differences in profiles when compared with the oranges and with each other. Temple's relative cholesterol content is twice that observed for the oranges and four times higher than found for the Temple x Kinnow. As observed in Tables II, III and IV, the relatively higher percentage of cholesterol in Temples is shown in all four sterol fractions. The Temple x Kinnow tangor is distinct in possessing the lowest percentage of cholesterol and stigmasterol and the highest percentage of sitosterol.

The distribution of sterols found in the sterol ester fraction is shown in Table II. In general the sequence of sterol distribution is β-sitosterol > campesterol > 24-ethylidene cholesterol > cholesterol > stigmasterol > 24-methylene cholesterol > brassicasterol. The two minor exceptions to this sequence are observed for Walker Early which possesses a higher percentage of cholesterol than 24-ethylidene cholesterol and the Valencia orange which shows similar percentages for stigmasterol and 24-methylene cholesterol. As was the case for the free sterol fraction, Walker Early, Parson Brown, Hamlin, Washington Navel and Pineapple possess essentially similar percentage profiles. The percentage of 24-ethylidene cholesterol found in Walker Early is relatively lower when compared with the other four oranges. Valencia differs from the other five oranges and the two tangors in possessing higher percentages of campesterol, stigmasterol and 24-methylene cholesterol. The distinguishing feature of the tangor group is observed in the Temple profile. Temple possesses the highest percentages of cholesterol and 24-ethylidene cholesterol when

compared with the Temple x Kinnow and the oranges.

Table III shows the percentage distribution of sterols in the SG fraction. The sequence of sterol distribution is β-sitosterol > campesterol > stigmasterol > 24-ethylidene cholesterol > cholesterol. Similarity in percentage distribution of sterols is again evident with Walker Early, Parson Brown, Hamlin, Washington Navel and Pineapple oranges. The relative percentage of cholesterol in the Temple tangor is approximately three times greater than found for the other cultivars.

The distribution of sterols in the ESG fraction is shown in Table IV. The relationships enumerated for oranges and tangors in the SG fraction are similar for the ESG fraction. While similar, comparative examination of Tables III and IV reveal some subtle differences. In all varieties the relative percentage of campesterol is ca. 1% higher in the SG fraction as compared with the ESG fraction. Conversely the relative percentage of β-sitosterol is ca. 1-2% lower in the SG fraction than ESG fraction.

Examination of Tables I-IV show both similarities and differences in the sterol composition of the eight cultivars. The sterol percentages of the four sterol fractions are distinct for each of the eight cultivars. Several general patterns emerge from these four tables. Sitosterol is the major sterol in all fractions; however the relative percentage of this sterol is greatest in the ESG fraction. Therefore for sitosterol the following percentage order in the four fractions is evident, viz., ESG > SG > SE > FS.

The order for campesterol is reversed to that of sitosterol, viz., FS > SE > SG > ESG. The order manifest by stigmasterol is different from both sitosterol and campesterol, i.e., FS > SG > ESG > SE. For cholesterol, SE > FS > ESG > SG is observed.

The two minor sterols, 24-methylene cholesterol and brassicasterol, are only detected in the FS and SE fractions. Brassicasterol and 24-methylene cholesterol could not be detected in the SG and ESG fractions. If these two sterols are present their percentages are well below 0.001%.

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The Effect of a Fat Free Diet on Esterified Monoenoic Fatty Acid Isomers in Rat Tissues[1]

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ABSTRACT

Rats were maintained 120-140 days on a normal diet (group 1) or one deficient in fatty acids (group 2). Isomer composition was determined of monoenoic fatty acids (16:1, 18:1) isolated from total lipids of heart, kidney, lung, brain and lumbar fat, and from separated neutral lipids and phospholipids of heart and kidney. Group 1: The number of major isomers of $C_{16:1}$ and $C_{18:1}$ was similar in all tissues but their proportions varied in different tissues and types of lipid. Group 2: The proportions of 16:1(*n*-7) increased and of other 16:1 isomers decreased in all tissues; 18:1(*n*-9) was increased at the expense of (*n*-7) in heart, to a lesser extent in kidney, and was little changed in lung, lumbar fat, or brain. The decrease in proportion of 18:1(*n*-7) was greatest in heart-muscle phospholipids. $C_{20:3}$ comprised 95% (*n*-9) and 5% (*n*-7) in heart and kidney lipids. The changes in group 2 probably represent the body's attempts to maintain lipids with the physical and chemical properties necessary to normal biological function.

INTRODUCTION

When the diet is deficient in essential fatty acids, the major changes in esterified fatty acids in the tissues are a decreased concentration of polyunsaturated fatty acids derived from the essential fatty acids $C_{18:2}$ (linoleic acid) and $C_{18:3}$ (linolenic acid) and increased concentration of $C_{16:1}$ (palmitoleic acid), $C_{18:1}$ (oleic acid) and $C_{20:3}$ (1). Fulco and Mead (2), using pooled tissues from fatty acid deficient rats, showed that $C_{20:3}$ (eicosatrienoic acid) is formed mainly by desaturation and chain lengthening from oleic acid, and that a small amount (<10%) is an isomer, differing in the position of the double bonds, derived from palmitoleic acid. Thus most fatty acids that are increased in this condition are members of the monoenoic fatty acid series or are derived from this series by chain elongation and desaturation. These changes probably represent attempts by the body to maintain lipids with the physical and chemical properties that are necessary for normal biological function.

The major monoenoic fatty acids (16:1 and 18:1) comprise a group of isomers that vary only in the position of the double bond. Despite the knowledge that these isomers, especially those of 18:1, make a significant contribution to the fatty acid composition of complex lipids, few studies have been made of their proportions in normal mammalian tissues (3-7) and the effect of dietary manipulation on these proportions (3,8). Sand et al. (8), who examined the esterified fatty acids from the total lipids of carcasses of rats fed a fat free diet, found that 16:1(*n*-7), the major 16:1 isomer, had increased at the expense of all other 16:1 isomers; the proportion of 18:1(*n*-7) also was slightly increased, with resultant decrease in 18:1(*n*-9). Similarly, Brockerhoff and Ackman (3) found 16:1(*n*-7) increased in the

TABLE I

Monoenoic Fatty Acid Isomer Composition of Control and Fat Free Diets[a]

Fatty acids[b]	Control diet	Fat free diet
Total 16:1	3.0	3.2
16:1(*n*-10)	2.8	23.3
16:1(*n*-9)	4.4	6.5
16:1(*n*-7)	89.2	66.8
16:1(*n*-5)	3.6	3.4
Total 18:1	31.2	27.1
18:1(*n*-10)	0.7	2.3
18:1(*n*-9)	96.0	92.3
18:1(*n*-7)	3.3	5.4

Each value is the mean of two separate isolations.

[a]Measured fatty acid content: control diet, 33.1 mg/g; fat free diet, 0.5 mg/g.

[b]Figures for total 16:1 and 18:1 are weight percentages of total fatty acids. Isomers are expressed as mole percentages of the total class; e.g., 16:1(*n*-10) is expressed as moles per cent of total 16:1.

[1]Nomenclature of fatty acids as in IUPAC-IUB Commission on Biochemical Nomenclature, "The nomenclature of lipids," J. Biol. Chem. 242:4845-49 (1967).

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TABLE II

Monoenoic Fatty Acid Isomers of Total Lipids in Organs of Rats Fed a Control (Group 1) or a Fat Free (Group 2) Diet[a]

Fatty acids[b]	Heart		Kidney		Lung		Brain		Lumbar fat	
	Group 1	Group 2	Group 1	Group 2	Group 1	Group 2	Group 1	Group 2	Group 1	Group 2
Total 16:1	0.7	6.7 ± 0.7	0.9	6.3 ± 0.9	3.2	8.2 ± 1.9	0.4	0.6 ± 0.4	3.5	11.2 ± 0.6
16:1(*n*-10)	12.6	2.5 ± 0.4	9.8 ± 1.1	4.5 ± 1.3	5.0 ± 0.5	2.9 ± 0.5	9.4	5.2 ± 0.9	2.1	1.4 ± 0.2
16:1(*n*-9)	6.9	4.0 ± 0.3	14.0 ± 1.0	4.4 ± 1.3	25.1 ± 1.1	10.3 ± 0.3	7.6	4.1 ± 0.2	6.1	3.3 ± 0.6
16:1(*n*-7)	74.8	92.5 ± 0.1	72.6 ± 1.6	90.2 ± 2.2	68.1 ± 0.4	86.0 ± 0.8	79.6	89.1 ± 0.8	89.9	94.5 ± 0.7
16:1(*n*-5)	5.7	1.1 ± 0.2	3.6 ± 0.4	1.0 ± 0.2	1.9 ± 0.2	0.8 ± 0.1	3.5	1.7 ± 0.6	1.9	0.6 ± 0.1
Total 18:1	11.6	34.2 ± 1.5	11.4	27.1 ± 1.0	23.4	32.3 ± 2.1	22.8	25.6 ± 0.5	37.9	54.4 ± 0.6
18:1(*n*-10)	1.8 ± 0.2	2.0 ± 0.2	1.1 ± 0.1	1.9 ± 0.2	1.2 ± 0.3	1.7 ± 0.4	1.1	1.2 ± 0.1	1.2 ± 0.3	2.1 ± 0.1
18:1(*n*-9)	71.2 ± 3.5	83.3 ± 2.4	79.2 ± 0.9	82.2 ± 1.0	87.4 ± 0.1	89.3 ± 0.7	84.2	82.1 ± 0.3	89.9 ± 0.9	90.1 ± 0.6
18:1(*n*-7)	27.0 ± 3.5	14.8 ± 2.2	19.6 ± 0.8	15.9 ± 1.1	11.4 ± 0.2	8.9 ± 1.0	14.7	16.6 ± 0.2	9.1 ± 0.6	7.8 ± 0.6

Each single value is the mean of two experiments. Mean ± SEM is for three experiments.

[a]Measured fatty acid content: control diet, 33.1 mg/g; fat free diet, 0.5 mg/g.

[b]Figures for total 16:1 and 18:1 are weight percentages of total fatty acids. Isomers are expressed as mole percentages of the total class; e.g., 16:1(*n*-10) is expressed as moles per cent of total 16:1.

depot fat of pigs fed a fat free diet, but in their studies the 18:1 isomers were little changed.

This study was undertaken to determine whether in the normal state the monoenoic fatty acid isomers of 16:1 and 18:1 differ in type and proportion in different tissues, and if a diet deficient in essential fatty acids alters the proportions of these isomers, affecting some tissues more than others. Such an effect, which would not be seen in examinations of total-carcass lipids, might reasonably be expected, in order to (i) maintain the proper proportions of unsaturated fatty acids with specific configurations, suitable to the special metabolic role of the various tissues, and (ii) supply precursors for longer-chain nonessential polyunsaturated fatty acids. In addition, one member of this latter group, eicosatrienoic acid ($C_{20:3}$), was examined to see whether its isomer composition differed in various tissues and, if so, whether this pattern correlated with differences in the parent isomer, 16:1(*n*-7) or 18:1(*n*-9).

EXPERIMENTAL PROCEDURES

Male Wistar rats weighing 90-110 g were used. For 120-140 days, group 1 (control) was maintained on Purina Laboratory Chow (Purina of Canada Ltd., Sherbrooke, Que.) and group 2 was fed a diet deficient in fatty acids (Nutritional Biochemicals Corp., Cleveland, Ohio). All other conditions were the same for both groups. After the animals had been killed, the brain (including cerebellum and rostral brain stem), heart, kidney, lung and lumbar fat were removed, washed with 0.85% NaCl in water and quickly frozen, and were stored at -20 C until extracted. In some cases tissues from two or three animals in one group were pooled to provide samples large enough for analysis. Total lipids were extracted from the tissues by homogenization in chloroform-methanol, 2:1, v/v (9). Neutral lipids (chiefly cholesterol, cholesterol esters, and triglycerides) were separated from phospholipids by chromatography on silicic acid (Unisil; Clarkson Chemical Co., Williamsport, Pa.) (10). Separation was complete as judged by phosphorus measurement (11) and thin layer chromatography (11,12). An average of 98% of applied phospholipid P was recovered. Using the methods described fully in earlier reports (4,13), total fatty acids were prepared and measured, pure monoenoic fatty acid methyl esters were isolated, and monoenoic fatty acid isomers were identified and quantified by periodate-permanganate oxidation and gas liquid chromatography of the dicarboxylic-acid dimethyl esters formed.

Relative amounts of each monoenoic fatty

TABLE III

Monoenoic Fatty Acid Isomers of Neutral Lipids and Phospholipids in Heart and Kidney[a]

	Heart				Kidney			
	Neutral lipid		Phospholipid		Neutral lipid		Phospholipid	
Fatty acids[b]	Group 1	Group 2	Group 1	Group 2	Group 1	Group 2	Group 1	Group 2
Total 16:1	2.9	7.8	0.6	3.7	2.6	6.6	1.0	5.1
16:1(*n*-10)	5.3	3.4	7.8	3.5	13.3	4.8	9.1	3.8
16:1(*n*-9)	5.3	2.6	3.7	2.3	8.8	3.2	17.3	1.7
16:1(*n*-7)	85.4	92.9	80.4	92.9	76.1	90.5	70.1	93.4
16:1(*n*-5)	4.1	1.2	8.2	1.3	2.2	1.7	3.8	1.3
Total 18:1	33.1	41.9	8.7	22.3	23.0	31.2	9.1	17.7
18:1(*n*-10)	1.5	2.5	1.4	2.1	2.1	2.1	1.4	2.0
18:1(*n*-9)	88.0	83.2	54.7	74.2	87.3	82.8	76.5	81.6
18:1(*n*-7)	10.7	14.3	44.0	23.6	10.7	15.2	22.1	17.2

Each value is the mean of two separate isolations.

[a]Measured fatty acid content: control diet, 33.1 mg/g; fat free diet, 0.5 mg/g.

[b]Figures for total 16:1 and 18:1 are weight percentages of total fatty acids. Isomers are expressed as mole percentages of the total class; e.g., 16:1(*n*-10) is expressed as moles per cent of total 16:1.

acid isomer are expressed as a percentage of the total class, abbreviated to moles per cent for convenience. For example, a sample of 18:1 yielding 70 moles of C_9 dicarboxylic acid and 30 moles of a C_{11} dicarboxylic acid would have consisted originally of 70% 18:1(*n*-9) and 30% 18:1(*n*-11).

RESULTS

The fat free diet contained very little fatty acid (footnote, Table I): the contribution to total fatty acids by $C_{18:2}$, $C_{18:3}$, and $C_{20:4}$ amounted to only 14.1%, compared with 35% in the control diet. The 18:1 isomer pattern in both diets was similar; that is, 18:1(*n*-9) (oleic acid) was the major isomer (Table I). The 16:1 isomer pattern was quite different, however: the fat free diet contained much more 16:1(*n*-10), and much less of the major isomer, 16:1(*n*-7).

Normal Diet (Group 1)

Of the 16:1 isomers, (*n*-7) was the major component in the total lipids of all normal tissues (Table II), especially lumbar fat. The (*n*-9) component was greater in lung than in kidney and was present in smaller, similar amounts in heart, brain and lumbar fat. There were distinct tissue-specific differences in the 18:1 isomer pattern: oleic acid (*n*-9) was the major isomer in all tissues, but the (*n*-7) content differed, decreasing in the order heart $>$ kidney $>$ brain $\geq$ lung $\geq$ lumbar fat.

Fat Free Diet (Group 2)

Total 16:1 and 18:1 were greatly increased in all tissues except brain (Table II). Not shown in the table are the marked increase in $C_{20:3}$ and concomitant decrease in $C_{20:4}$ in heart, kidney, and lung; the same pattern, but of minor extent, was observed in brain, and there was no detectable change in the low levels ($<$1.0%) of these fatty acids in lumbar fat. The major alteration in the 16:1 isomer pattern was an increase in the proportion of (*n*-7) at the expense of all other isomers. In the 18:1 group the proportion of (*n*-9) increased at the expense of (*n*-7) in heart and to a slight extent in kidney and lung but there was little change in brain or lumbar fat. Analysis of the 20:1 fraction in brain (the only tissue containing amounts sufficient for analysis) showed little difference from values in the control rats.

These changes in the tissues of the group 2 rats could be accounted for by an increase in triglycerides of an isomer composition similar to that of lumbar fat; that is, if the change were toward the proportions in lumbar fat. Direct measurement of the lipid weight and phospholipid content of these organs showed very little increase in the former ($<$5%), apparently due to increase in neutral lipid (mean values for neutral lipid in control tissues: heart, 30%; kidney, 23% of lipid wt). Accordingly a major shift in isomer proportions in either neutral lipids or phospholipids or both seemed necessary to explain the changes in the total-lipid isomer pattern in heart and to a lesser extent in kidney.

Neutral lipids and phospholipids were isolated from heart and kidney tissues and their monoenoic fatty acid isomer patterns were determined. In both lipid types the diet-induced change in 16:1 isomers was similar: the proportion of (*n*-7) had increased at the ex-

pense of all other 16:1 isomers (Table III). In the 18:1 group of isomers (*n*-7) was slightly greater in heart and kidney neutral lipids; it was slightly less in kidney phospholipids and markedly decreased in heart phospholipids.

The fat free diet had increased the amount of $C_{20:3}$ in heart, kidney, and lung. This acid was isolated by argentation thin layer chromatography and preparative gas liquid chromatography (4) from total lipids of heart and kidney; after hydrogenation (14) the fatty acid chromatographed identically with $C_{20:0}$. It was oxidized with periodate-permanganate and the dicarboxylic acid fragments were examined. Assuming a methylene-interrupted pattern of double bonds (2), 20:3 from both kidney and heart consisted of 94-95% (*n*-9) and 5-6% (*n*-7).

DISCUSSION

In the control animals (*n*-7) was the major 16:1 isomer in all tissues and 16:1(*n*-9) was present in greatest proportions in lung; (*n*-9) was the major 18:1 isomer in all tissues, and the 18:1(*n*-7) contribution decreased in the order heart > kidney > brain ≥ lung ≥ lumbar fat.

In group 2 rats all tissues except brain contained increased amounts of 16:1, due chiefly to increase in (*n*-7); and although total 16:1 in brain did not alter significantly the (*n*-7) component there, too, was increased. These results agree with those for total-carcass lipids (8) and pig depot fat (3). The increased 16:1(*n*-7) component in all of the tissues examined could have been derived from the diet only if there was selective uptake of this isomer at the expense of (*n*-10): the diet contained relatively large amounts of (*n*-10) but the tissues contained relatively little. It is more likely that the fat free diet increased the endogenous synthesis of 16:1(*n*-7) as has been shown in liver microsomes (15), and that this accounts for the increased proportion of 16:1(*n*-7) in tissue.

Although the total 18:1 fraction was markedly increased in all tissues except brain in the group 2 rats there were major alterations in the isomer pattern in only the heart and minor changes in the kidney, consisting in altered proportions of 18:1(*n*-7) and (*n*-9). As the total 18:1 fraction increased, 18:1(*n*-7) increased in *amount;* but its *proportion* decreased due to increase in the proportion of 18:1(*n*-9). The change in 18:1 isomer composition in heart muscle and to a lesser extent in kidney was due to decrease in the proportion of 18:1(*n*-7) in the phospholipid fraction and only slight increase in the neutral-lipid fraction.

The increase in 18:1(*n*-7) during a fat free diet observed by Sand et al. (8) in total-carcass lipids was observed in only heart and kidney neutral lipids in this study (Table III); it was not observed in the whole-organ lipids, due to the relatively greater decrease in 18:1(*n*-7) in the phospholipid fraction. That the results obtained in this study differ from those of Sand may mean that certain tissues which were not individually studied respond to the diet by increasing the amount of 18:1(*n*-7). Alternatively, the age of the animals may be concerned. (The rats used by Sand et al. (8) were 25 days old, whereas those in the present study weighed 90-110 g and therefore were older.) Diet-induced fatty acid changes in complex lipids develop more rapidly in young than in older animals (16), and alterations in the monoenoic fatty acid isomers in their tissues may differ.

During the fat free regimen, eicosatrienoic acid ($C_{20:3}$) accumulated in heart and kidney, and to a lesser extent in lung and brain, and the levels of arachidonic acid ($C_{20:4}$) fell. The $C_{20:3}$ was chiefly the (*n*-9) isomer in heart and kidney, the only two tissues containing quantities sufficient for this examination; this probably reflects the larger amount of 18:1(*n*-9) than of 16:1(*n*-7) available for chain elongation and desaturation. Similarly, Fulco and Mead (2) obtained about 93% of the (*n*-9) isomer in the 20:3 fraction isolated from pooled organs of fat deficient rats. The finding in both studies that $C_{20:3}$ was predominantly (*n*-9) supports the suggestion (17) that the presence of nine carbon atoms between the carboxyl group and the first double bond is an important factor in determining whether further desaturation takes place. Linoleic acid (18:2 Δ 9,12), linolenic acid (18:2 Δ 9,12,15) and oleic acid (18:1 Δ 9) have nine carbons between the carboxyl group and the first double bond, whereas 18:1(*n*-7), which is not further desaturated to the same degree, has 11 such carbon atoms.

The greatest changes in monoenoic isomer proportions were in heart-muscle lipids, particularly their phospholipids. Phospholipids form an integral part of all mammalian membrane systems (18), and diet-induced alterations in their fatty acid content, with consequent changes in physical properties, may have pathologic significance. Examples of altered membrane properties in fatty acid deficiency are increases in erythrocyte fragility (19) and capillary permeability (20). In rats deficient in essential fatty acids, enlarged mitochondria with disorganized cristae have been observed in heart (21), and an abnormal electrocardiogram (notching of the QRS complex) is a consistent feature (22). The altered ratios of the monoenoic fatty acid isomers of heart-membrane

phospholipids may contribute to these effects.

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Isolation and Identification of Cholesterol α-Oxide and Other Minor Sterols in Human Serum[1]

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ABSTRACT

The isolation and identification of cholesterol α-oxide, coprostanol, β-sitosterol, cholest-4-en-3-one and cholesta-4, 6-dien-3-one from human serum are reported. Compounds were isolated by thin layer chromatography and were identified by gas liquid chromatography and gas chromatography-mass spectrometry (GC-MS). Data for standard sterols are also reported. The possible origins of these minor components and the significance of their presence are discussed.

INTRODUCTION

The occurrence of sterols other than cholesterol in serum has been reported in several studies. Nakanishi et al. (1) estimated lathosterol (5α-cholest-7-en-3 β-ol) in serum as 0.6% of cholesterol. Koehler and Hill (2) reported the presence of sterols which were fast-acting with Liebermann reagent; average concentrations were estimated to be 3.4 mg free sterol/100 ml serum and 16.5 mg sterol ester/100 ml serum. The authors attributed the reaction mainly to cholesta-5,7-dien-3β-ol. Chattopadhyay and Mosbach (3) described a method for the determination of cholestanol in serum and gave human values as 0.716-0.769% of the total sterol present.

In recent years there have been further studies on minor sterols present in human serum. Claude and Beaumont (4,5) have reported the presence of desmosterol, cholesta-5,7-dien-3β-ol, coprostanol and cholesta-3,5-dien-7-one; they identified the compounds mainly by thin layer chromatography (TLC) but also used gas liquid chromatography (GLC). Miettinen (6) has identified various lanosterol and methyl sterol precursors in serum, including lanosterol, dihydrolanosterol (5α-lanost-8(9)-en-3β-ol), 4α-methyl-5α-cholest-7-en-3β-ol and 4α-methyl-5α-cholest-8(9)-en-3β-ol. Coprostanol and 24β-ethylcoprostanol were also identified. The sterols were identified by TLC, GLC, and GC-MS. We have previously reported

[1]Throughout the paper the following nomenclature is used: cholesterol α-oxide, cholesterol-5α, 6α-epoxide; coprostanol, 5β-cholestan-3β-ol; cholestanol, 5α-cholestan-3β-ol; dihydrolanosterol, 5α-lanost-8(9)-en-3β-ol; β-Sitosterol, 24β-ethylcholest-5-en-3β-ol; cholesterol, cholest-5-en-3β-ol; coprostanone, 5β-cholestan-3-one.

TABLE I

Mobilities for Standard Compounds and Their TMS Ethers on Silver Nitrate–Silica Gel G Layers

Compound	Free sterols[a]		TMS[b] ethers[c]	
	Rf	Rchol[d]	Rf	Rchol[d]
Cholesterol	0.44	1.00	0.47	1.00
Coprostanol	0.52	1.18	0.74	1.58
Cholestanol	0.43	0.98	0.45	0.96
Desmosterol	0.35	0.79	0.31	0.66
Lanosterol	0.53	1.20	0.57	1.21
Dihydrolanosterol	0.53	1.20	0.66	1.42
β-Sitosterol	0.45	1.02	0.40	0.85
25-Hydroxycholesterol	0.15	0.34	0.31[e]	0.66
Cholesta-4,6-dien-3-one	0.61	1.39	---	---
Cholest-4-en-3-one	0.62	1.41	---	---
Coprostanone	0.73	1.66	---	---
Cholesterol α-oxide	0.20	0.45	0.12	0.25
5α-Cholestane-3β, 5-diol	0.09	0.20	0.18[f]	0.38

[a]Solvent system: Chloroform/acetone 9/1.
[b]Trimethylsilyl.
[c]Solvent system: Hexane/benzene 4/1.
[d]Mobility relative to cholesterol or its TMS ether.
[e]Bis TMS.
[f]Mono TMS.

TABLE II

Retention Index Values for Standard Compounds and Their TMS[a] Ethers

Compound	1% OV-1, 235 C		1% OV-17, 235 C	
	$I_{Free\ sterol}$	I_{TMS}[a]	$I_{Free\ sterol}$	I_{TMS}[a]
Cholesterol	3040	3110	3300	3220
Coprostanol	2990	3030	3250	3120
Cholestanol	3055	3120	3310	3235
Desmosterol	3070	3155	3415	3300
Lanosterol	3230	3270	3510	3405
Dihydrolanosterol	3195	3250	3445	3330
β-Sitosterol	3210	3285	3495	3420
25-Hydroxycholesterol	3215	3280[b]	3590	3520[b]
		3370[c]		3470[c]
Cholesta-4,6-dien-3-one	3170	---	3495	---
Cholest-4-en-3-one	3150	---	3465	---
Coprostanone	3035	---	3305	---
Cholesterol α-oxide	3160	3255	3485	3395
5α-Cholestane-3β, 5-diol	3200	3245[b]	3535	3425[b]
		3195[c]		3480[c]

[a]Trimethylsilyl.
[b]Mono TMS.
[c]Bis TMS.

(7) the identification of dihydrolanosterol, lanosterol, desmosterol and cholesta-3,5-dien-7-one. The present work reports further identification of minor sterols found in human serum and discusses the significance of their presence.

MATERIALS AND METHODS

Extraction and Fractionation of Sterols

Serum, 20 ml, was mixed with chloroform-methanol, 400 ml 2:1 v/v (8,9), and left at room temperature for 3 hr with occasional shaking. The mixture was filtered to remove precipitated protein and equilibrated with 0.9% (w/v) sodium chloride solution overnight at 4 C. The chloroform-methanol layer was removed, dried with sodium sulphate and evaporated to dryness to give the total lipid extract.

Fractionation into lipid classes was achieved

TABLE III

Chromatographic Data for Cholesterol α-Oxide

Compound	Retention Index	
	1% OV-1, 235 C	1% OV-17, 235 C
Isolated compound	3160	3485
Isolated compound TMS[a] ether	3240	3405
Isolated compound trifluoroacetate	3080	3225
Isolated compound acetate	3305	3610
Cholesterol α -oxice	3160	3485
Cholesterol α-oxide TMS[a] ether	3225	3395
Cholesterol α-oxide trifluoroacetate	3075	3225
Cholesterol α-oxide acetate	3305	3610
$LiAlH_4$ reduction		
Isolated compound, reduced	3200	3530
Isolated compound, reduced: mono TMS[a] ether	3245	3425
Isolated compound, reduced: bis TMS[a] ether	3195	3480
5α-Cholestane-3β, 5-diol	3200	3535
5α-Cholestane-3β, 5-diol mono TMS[a] ether	3245	3425
5α-Cholestane-3β, 5-diol bis TMS[a] ether	3195	3480

[a]Trimethylsilyl.

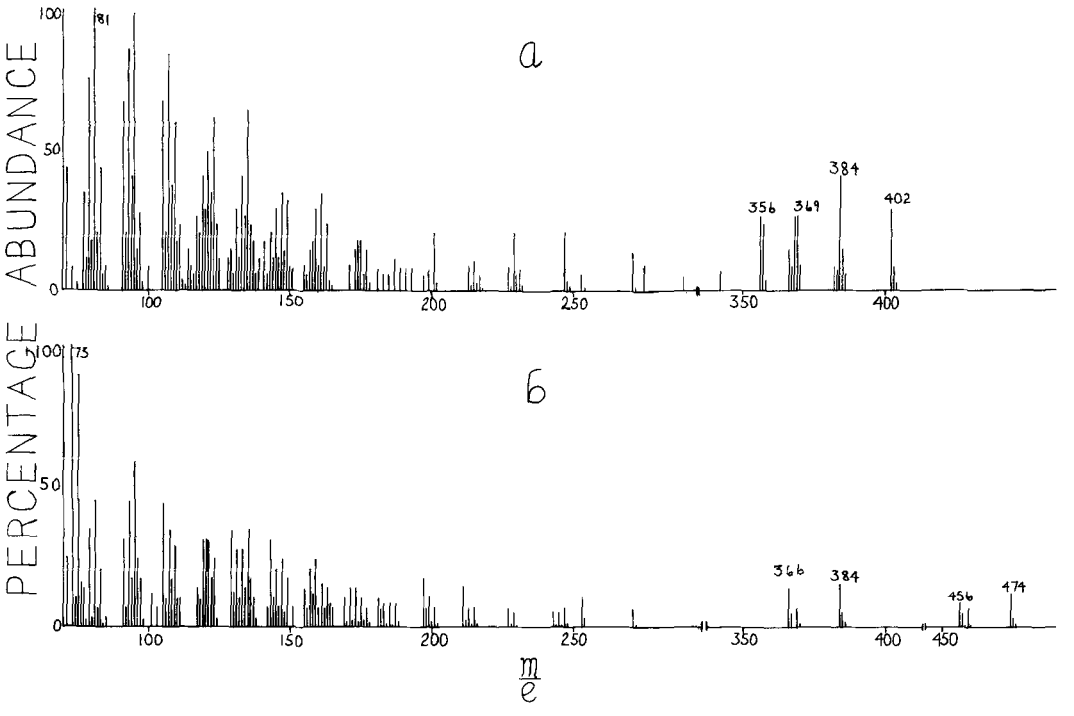

FIG. 1. Mass spectra of (a) cholesterol α-oxide and (b) cholesterol α-oxide TMS ether, isolated from human serum. Recording conditions as described in the text.

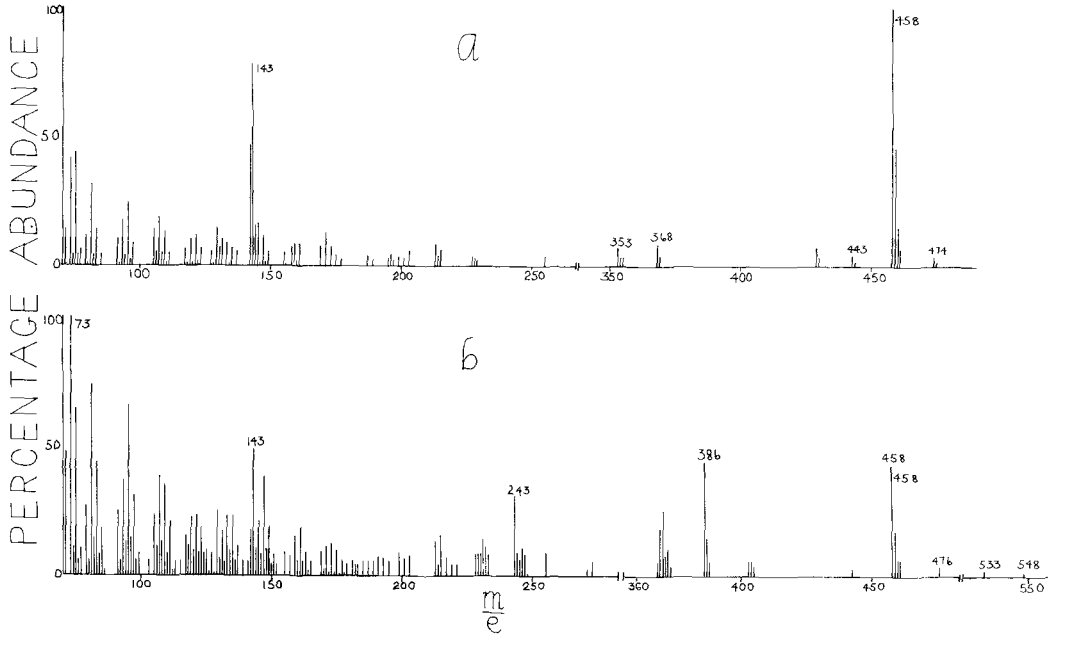

FIG. 2. Mass spectra of (a) mono TMS ether and (b) bis TMS ether of the product of $LiAlH_4$ reduction of cholesterol α-oxide isolated from human serum. Recording conditions as described in the text.

by preparative TLC on Silica Gel G. Plates were prepared by slurrying 30 g Silica Gel G (E. Merck AG, Darmstadt) with 60 ml water, and spreading 1.0 mm thick layers using a commercial spreader (Shandon Scientific Company Ltd., London). They were dried at room temperature and activated by heating for 3 hr at 120 C. The lipid extract was first run in hexane, which separated the hydrocarbons to the solvent front. The plates were then dried, the top few centimeters of the layer cut off and the plates re-run in benzene-chloroform (4:1 v/v) which separated the sterol esters, triglycerides and free sterols. The sterol esters were hydrolyzed using 2% alcoholic potassium hydroxide, 0.5 ml/mg sterol ester (10), at 40 C for 2 hr. Petroleum ether, bp 60-80 C, 10 vol, was then added and the mixture was extracted with an equal volume of distilled water. The organic layer was dried with sodium sulphate and taken to dryness to give sterols resulting from the hydrolysis of esters.

The sterols were fractionated by TLC on silver nitrate-impregnated Silica Gel G layers (11). These were prepared by dissolving 1 g silver nitrate in 60 ml water and slurrying this with 30 g Silica Gel G. The plates (0.5 mm) were then spread and the layers dried at room temperature in the dark and activated by heating to 80 C for 2 hr.

The sterols were run both as free sterols in chloroform-acetone 9:1 (v/v) and as trimethylsilyl ethers (TMS ethers) in hexane-benzene (4:1 v/v) (12). Mobilities of standard compounds are given in Table I. As can be seen the TMS ethers give better separations, but this advantage is partially offset by their tendency to decompose in the presence of traces of acids and thus to "tail" on the plate.

The TMS ethers were formed by treating the sterols with hexamethyldisilazane (HMDS) and trimethylchlorosilane (TMCS) at room temperature overnight. Excess reagents were blown off under nitrogen and the products dissolved in hexane.

After fractionation by TLC the sterols were analyzed by GLC using a Perkin-Elmer F.11 dual column flame ionization chromatograph. The 6 ft glass columns were packed with 1% OV-1 and 1% OV-17, both on 100/120 mesh Gas Chrom Q (Applied Science Laboratories Ltd., State College, Pa). The oven temperature was 235 C, injection block 250 C, and the carrier gas (N_2) flow rate was 50 ml/min. Sterols were chromatographed both as the free sterols and as TMS ethers. Retention indices for some standard sterols are given in Table II.

Suitable fractions were submitted to GC-MS using an LKB 9000 gas chromatograph-mass

TABLE IV

Chromatographic Data for β-Sitosterol and Coprostanol Isolated From Human Serum

	Thin layer chromatography				Gas liquid chromatography		
	Free Sterol[a]		TMS[b] ether[c]			Retention Index	
Compound	Rf	Rchol[d]	Rf	Rchol[d]	Compound	1% OV-1, 235 C	1% OV-17, 235 C
β-Sitosterol	0.53	1.00	0.52	0.92	β-Sitosterol	3210	3495
Coprostanol	0.71	1.22	0.90	1.64	β-Sitosterol TMS[b] ether	3285	3420
					Coprostanol	2990	3250
					Coprostanol TMS[b] ether	3030	3120

[a]Solvent system chloroform-acetone 9:1.
[b]Trimethylsilyl.
[c]Solvent system hexane-benzene 4:1.
[d]Mobility relative to cholesterol or its TMS ether.

TABLE V

Mass Spectral Data for β-Sitosterol and Coprostanol Isolated From Human Serum

Compound	Parent ion (M)	Base peak	Other ions
β-Sitosterol TMS[a] ether	486(18%)[b]	129[c]	95(90%); 357[d](62%); 396[e](55%); 97(53%); 107(48%); 93(45%); 121(35%) 381[f](25%); 255[g](21%); 471[h](11%)
Coprostanol TMS[a] ether	460(2%)	73	370[e](60%); 109(40%); 111(38%); 147(38%) 107(33%); 149(29%); 215(28%); 221(16%); 257[g](12%);355[f](9%)

[a]Trimethylsilyl.
[b]Figures in parenthesis are percentage abundances.
[c]$[(CH_3)_3-Si-O-CH-CH=CH_2]^+$
[d]M-129
[e]M-90 ($(CH_3)_3-SiOH$)
[f]M-105 (90,15)
[g]M-90-sidechain
[h]M-15 (CH_3)

spectrometer. Samples were separated using either a 10 ft column at 250 C or a 6 ft column at 235 C. Both 1% OV-1 and 1% OV-17 were used, as appropriate. Mass spectrometry was effected with a source temperature of 290 C, ionizing voltage 70 ev, accelerating voltage 3.5 kv, electron multiplier setting 2.7 kv.

RESULTS

Identification of Sterols

In many extracts the presence of a sterol more polar than cholesterol was noted in both the free sterol and sterol ester fractions. The chromatographic data are shown in Table III, and the mass spectra of the free sterol and TMS ether in Figure 1.

The formation of a bis TMS ether was attempted using bistrimethylsilylacetamide (BSA)-trimethylsilylimidazole (TSIM)-TMCS, 3:3:2 (13). The reagents were added to the sterol and the mixture was kept at 60 C for 66 hr. The excess reagents were then blown off under nitrogen and the residue was taken up in hexane for GLC. Only the mono TMS ether was obtained. This method of silylation will form TMS ethers of even the most highly hindered hydroxyl groups in the nucleus (14). Thus the failure to form a bis TMS ether suggested that the compound contained a ketone or epoxide group rather than a second hydroxyl group. The sterol was therefore reduced using lithium aluminium hydride and was found to be altered (Table III). Trimethylsilylation of the resulting compound under the usual conditions formed only a mono TMS ether (shown by retention increments on GLC and its mass spectrum), but on treatment with BSA/TSIM/TMCS, 3:3:2, as described above a bis TMS ether was formed (see Table III and Fig. 2 for mass spectra). Thus the reduction product contained a hindered hydroxyl group, e.g., 5α,5β,14α; comparison with standards suggested that it was 5α-cholestane-3β,5-diol. This was confirmed by comparison of GLC and mass spectral data with those of authentic 5α-cholestane-3β,5-diol (Table III). The identity of the original polar sterol as cholesterol α-oxide (15) was confirmed by direct comparison of its chromatographic and mass spectrometric properties with those of authentic material.

β-Sitosterol was found in many extracts and was identified by comparison of the TLC, GLC and mass spectral data with those of a reference sample of β-sitosterol (Tables IV and V).

Coprostanol was found in every extract studied and was also identified by comparison of TLC, GLC and mass spectral behavior with that of authentic coprostanol (Tables IV and V). Standard mass spectral data of β-sitosterol TMS ether and coprostanol TMS ether have been published (16).

Cholest-4-en-3-one and cholesta-4,6-dien-3-one were found running above cholesterol on TLC. Cholest-4-en-3-one has been identified on the basis of TLC, GLC and mass spectral data and its UV absorption. Standard mass spectral data have been published (34). Cholesta-4,6-dien-3-one has been identified on the basis of chromatographic data and of its UV absorption (Table VI).

DISCUSSION

Occurrence and Significance of Cholesterol α-Oxide

Cholesterol α-oxide has not previously been identified from a natural source, although it has

been found among the autoxidation products of cholesterol in one study (17) but not in others (18-20). The possibility that cholesterol α-oxide could have arisen from the autoxidation of cholesterol during the extraction and separation procedures in this study has been rejected for the following reasons: (a) The quantities of cholesterol α-oxide in the extracts did not increase with time; (b) extracts of different sera, worked up in parallel under the same conditions, contained widely differing amounts of cholesterol α-oxide; (c) there is no evidence for other autoxidation products of cholesterol, e.g., 7-hydroxycholesterols, 7-ketocholesterol, etc., in the extracts; (d) cholesterol α-oxide has never been detected in samples of cholesterol similarly treated.

Steroid epoxides are rare in nature, the principal examples being several toad poisons (21).

Recent studies have shown that after intubation of cholesterol α-oxide emulsion in the rat, no trace of cholesterol α-oxide was found in the blood or tissues (22) and that cholesterol α-oxide is metabolized to 5 α-cholestane-3β, 5,6β-triol in the gastro-intestinal tract (23). The presence of cholesterol α-oxide in, and the absence of the triol from, human serum suggest that cholesterol α-oxide is not metabolized in this manner in humans.

The quantity of cholesterol α-oxide isolated in the extracts ranged from none to 4,500 μg/100 ml serum.

The known clinical data, cholesterol levels and cholesterol α-oxide levels for the extracts are given in Table VII.

Extracts containing no cholesterol α-oxide (or only a trace thereof) came mainly from normal volunteers and patients with normal lipid patterns. Higher levels of cholesterol α-oxide were found in patients with varying degrees of hypercholesterolemia and also varying degrees of atherosclerosis. It appears that there may be a relation between the severity of atherosclerosis and the level of cholesterol α-oxide in serum. However this will require further investigation.

Occurrence and Significance of β-Sitosterol and Coprostanol

β-Sitosterol was identified in many extracts, both as the free sterol and the sterol ester, in quantities ranging from 10-400 μg/100 ml serum. It undoubtedly originates from dietary sources by absorption from the intestine.

The absorbability of β-sitosterol has been the subject of several investigations. Schoenheimer (24) first studied it in experimental animals in 1929 and concluded on the basis of liver sterol

TABLE VI

Chromatographic and Spectral Data for Cholest-4-en-3-one and Cholesta-4,6-dien-3-one Isolated from Human Serum

Compound	Thin layer chromatography[a]		Gas liquid chromatography[b]		UV		Mass Spec[c]	
	Rf	R_{chol}	I_{ov-1}	I_{ov-17}	λ max(observed)	λ max(standard)	M^+	Base peak
Cholesta-4-en-3-one	0.75	1.45	3155	3465	241nm	242nm	384	124
Cholesta-4,6-dien-3-one	0.70	1.35	3175	3485	287nm	286nm	---	---

[a]Solvent system used was chloroform-acetone 9:1.
[b]Temperature 235 C.
[c]Other prominent ions occurred at $\frac{m}{e}$: 95 (33%); 229 (31%); 107 (28%); 135 (24%); 147 (22%); 261 (16%); 342 (11%); 369 (4%).

TABLE VII

Levels of Cholesterol α-Oxide Found in Serum Extracts

Clinical condition	Serum cholesterol,[a] mg/100 ml serum	Cholesterol α-oxide levels,[b] μ/100 ml serum
Normal	114	0
Normal	214	0
Normal	174	trace <5
Peptic ulcer	184	25
Mild high blood pressure	300	30
Collagen disease	263	50
High blood pressure	342	150
Type II hypercholesterolemia	454	250
Unknown	284	500
Hypercholesterolemia	358	1,500
Type II hypercholesterolemia	316	3,250
Hypercholesterolemia, pooled samples	356	4,550

[a]Measured by Pearson method (35).
[b]Estimated by gas liquid chromatography.

concentrations that the sterol was not absorbed. Gould (25) showed that absorption occurred to an extent of 10% after feeding tritiated β-sitosterol to terminal patients, and he has reported β-sitosterol levels in serum of 4.5-38.6 μg/100 ml after administration of a single dose of [^{3}H]-β-sitosterol (26).

The metabolism of β-sitosterol has recently been extensively studied by Salen et al. (27) and they have reported 5% or less absorption of dietary β-sitosterol with serum levels ranging from 0.30 to 1.02 mg/100 ml, in patients ingesting amounts of β-sitosterol typical of the American diet. These levels are much higher than we have observed and may reflect a difference between American and British diets.

Coprostanol was found in all extracts, only as the free sterol, in quantities ranging from 10-20 μg/100 ml serum. Cholesterol is converted to coprostanol in the intestine by bacterial action and its presence in serum indicates that a small amount of absorption takes place. The levels found are in agreement with those reported by Miettinen (6) in the serum of obese patients, viz., 7-24 μg coprostanol/100 ml serum. One extract however contained a much higher level of coprostanol (~200 μg/100 ml serum) but no reason for this could be found. Salen et al. (27) reported individual variations in the absorption of β-sitosterol and this higher level may merely represent an individual variation in the absorption of coprostanol.

The fact that coprostanol has been found only as the free sterol is in accord with the work of Rosenfeld et al. (28). They showed by feeding [^{14}C]-coprostanol that radioactivity was recovered only from the free coprostanol fraction and that no radioactivity was found in the ester fraction. They have also shown (29) that administered epicoprostanol becomes partly esterified and they conclude that for esterification to occur an equatorial conformation of the 3-hydroxyl group is required.

Occurrence and Significance of Cholest-4-en-3-one and Cholesta-4,6-Dien-3-one

Cholest-4-en-3-one was found in the free sterol fraction of all the extracts in quantities ranging from 10-30 μg/100 ml serum, while cholesta-4, 6-dien-3-one was found in the free sterol fraction in about half the extracts studies in quantities ranging from 10-20 μg/100 ml serum.

Little is known of the source or functions of cholesta-4, 6-dien-3-one but cholest-4-en-3-one has been shown to reduce serum cholesterol levels (30,31) in rats. However it was also found to be highly toxic. Cholest-4-en-3-one has also been demonstrated fairly conclusively to be an intermediate in the formation of cholestanol from cholesterol (32,33). Its presence in serum in small amounts could arise from this source.

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Metabolism of Phospholipid in Mammary Gland: I. The Supply of Phospholipid for Milk Synthesis in the Rat and Goat

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ABSTRACT

Data presented in this study demonstrate that under normal physiological conditions, milk phospholipids in the rat and the goat originate predominantly, if not totally, by de novo synthesis within the mammary gland. Evidence to support this has been obtained for the goat by measurement of P^{32}-phosphate incorporation into milk phospholipids, and in the rat by measurement of P^{32}-phosphate incorporation and by feeding radioactive phospholipid to measure the incorporation of serum phospholipids into milk. The latter experiment showed that the fatty acid portion of the dietary phospholipid can readily be utilized by the mammary gland for triglyceride synthesis, but that the contribution of the serum phospholipid "backbone" to milk phospholipid is minimal.

INTRODUCTION

Previous data on the role of serum phospholipids in milk production by mammary gland have indicated that the circulating serum phospholipids play no direct part in the secretion of milk phospholipids by this tissue (1-4). Arteriovenous difference studies across mammary gland (1,2) indicate no significant uptake of phospholipid.

However a consideration of serum and milk phospholipid levels and of blood circulation time demonstrates that all milk phospholipids could be derived from blood supplies without a measurable drop in the level of serum phospholipids across the gland. For the lactating rat the levels of phospholipid in milk and serum were determined (5) as 130 mg/100 ml and 150 mg/100 ml respectively. If it is assumed that 10% of the total blood flow passes through the mammary gland, then from the cardiac output (35 ml/min [6]), total blood volume (6.7% body weight [7]), hematocrit of 0.45 (5), and the milk production rate (26 ml per day [8]), it can be calculated that the mean mammary serum flow to milk yield in a 300 g rat is approximately 87:1. From this datum, the arteriovenous difference across the mammary gland if the serum phospholipid provided all of the milk phospholipid would be 1.00%.

The corresponding data for the lactating goat are: serum phospholipid 160 mg/100 ml (9), milk phospholipid 37 mg/100 ml (10), and mean mammary plasma flow to milk yield 353:1 (9). By the same calculation as above, the corresponding arterio-venous difference if the serum provided all of the milk phospholipid would be 0.07%.

It has been suggested (3) that serum phospholipids are stabilizers of serum lipoproteins, but can also act as carriers of specific fatty acids to the mammary gland. However, experiments with P^{32}-labelled lipoproteins have demonstrated that the mammary glands of the cow (3) and rabbit (4) take up serum phospholipids. In the rabbit, no activity was recovered in the milk (4). Instead, the absorbed phospholipid was broken down in the tissue.

This study represents an attempt to measure the relative contributions of serum phospholipid and de novo synthesis within the mammary gland to milk phospholipid in the lactating rat and goat.

MATERIALS AND METHODS

Animals

All rats used were lactating females (250-400 g), of Sprague-Dawley strain, each nursing six pups, and experiments were performed between 10-18 days post partum. The rats were maintained on normal laboratory diet and water ad lib. The young were left with the mother and allowed to suckle at will for the duration of the experiment.

The goat used was from the department herd and was producing 1200 ml of milk daily at the start of the experiment. The animal was maintained on a normal hay-grain ration and water ad lib.

Radioactive Isotopes

Radioactive phosphorus (Carrier-free P^{32}-orthophosphoric acid in 0.02N HCl) was obtained from New England Nuclear, Boston, Mass. The acidity of the solution was neutralized before use. U-C^{14}-phosphatidyl-choline 1800 $\mu c/\mu$mole) was also obtained from New England Nuclear (Cat. No. 588, batch 526-061). The isotope contained 1.8% of the

activity as free fatty acid, and 2% as lysolecithin. It was used without further purification.

Administration of Isotope

Radioactive phosphorus was administered intravenously. In the experiment with rats the first animal was given 25 μc of the isotope and the second rat 10 μc. In each case the isotope was administered in 0.2 ml saline. In the goat experiment the animal was given 200 μc of the radioactive phosphorus injected in 4 ml of dilute phosphate buffer pH 7.

Uniformly labelled C^{14}-phosphatidyl choline (5 μc) was administered to one rat by gavage in 0.6 ml corn oil.

Collection of Samples

Blood samples were collected at various times after the administration of the isotopes—in the rat from the tail under ether anesthetic, and in the goat by venipuncture. Milk samples were collected from the goat by hand expression. In the rat, milk samples were obtained under slight suction after intramuscular injection of 0.1 USP unit oxytocin, usually at the same time as blood collection.

Extraction of Lipids

Rat Experiments: Cream and skim milk fractions were obtained from whole milk (0.5-2.0 ml) by centrifugation (2.0×10^4 x g min.). The cream samples were dissolved in 20 volumes of chloroform-methanol (2:1 v/v), and the skim-milk fractions added to 4 volumes of chloroform-methanol (1:3 v/v) to obtain a one-phase system. The skim-milk samples were then taken to dryness below 50 C under nitrogen (using acetone to remove water by co-evaporation), and the lipids dissolved in chloroform methanol (2:1 v/v).

Samples of blood serum, prepared from whole blood by centrifugation at 4.0×10^4 x g min, were treated in the same way as skim-milk fractions.

Goat experiment: The milk samples (40 ml) were cooled in ice, then centrifuged at 3.7×10^4 g/min, and the skim-milk drained from under the fat layer. Serum samples were prepared from whole blood as described above.

Skim-milk, cream and sera were extracted by the Roese-Gottlieb procedure (11). The extracts were evaporated to dryness under vacuum at 38 C, and the lipids taken up in a small quantity of chloroform.

Separation of Lipid Components

The various lipid classes in the extracts were separated in two ways. For the rat extracts,

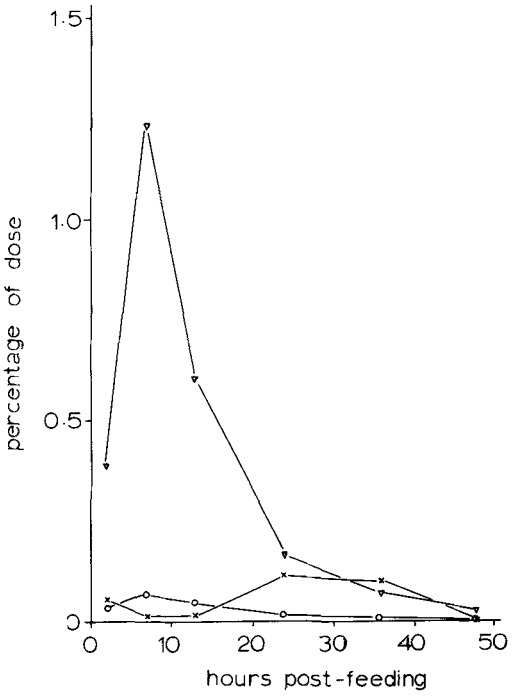

FIG. 1. Serum and milk radioactivity levels after $U^{14}C$-phosphatidyl choline feeding. The lactating rat was fed 5 μc of isotope in 0.6 ml of corn oil by gavage. Key: -▽- and -○- radioactivity equivalent to 1 ml of whole milk in cream and skim respectively, -x- total radioactivity per ml serum.

where the amount of lipid available was small, separation was effected by thin layer chromatography on Silica Gel F_{254} plates (E. Merck, Darmstadt, Germany), using for neutral lipids the solvent system: petroleum ether (boiling range 30-60 C): diethyl ether: acetic acid (70:30:1 v/v/v), and for polar lipids the two-dimensional system of Parsons and Patton (12). (First solvent, chloroform-methanol-water-ammonia [130:70:8:0.5 by vol]; second solvent, chloroform-acetone-methanol-acetic acid-water [100:40:20:20:10, v/v]). The lipids were extracted from the silicic acid after separation by scraping the gel off the plate and extracting several times with small quantities of polar solvents, e.g., diethyl ether for neutral lipids, methanol for polar lipids.

In some instances the location of the C^{14}-radioactivity in lipid classes was determined by autoradiography (13).

For the goat extracts, where larger quantities of material were available, extracts were chromatographed on silicic acid columns (14) to separate the phospholipids from neutral lipids. Portions of the phospholipid from the skim-milk and cream fractions at 31 hr were chro-

TABLE I

Radioactive Composition of Lipid Fractions -U-^{14}C-Phosphatidyl Choline Experiment

Sample	Percentage total radioactivity in each fraction				
	PL[a]	DG	Chol + FFA	TG	CE
Serum 2-7 hr	51.0	12.0	11.0	19.0	8.0
Serum 24-36 hr	44.0	10.0	35.0	3.0	7.0
Cream 7 hr	0.6	2.4	1.0	93.9	2.1
Skim 7 hr	2.2	2.5	1.9	92.4	0.7

[a]Abbreviations: PL, phospholipid; DG, diglyceride; Chol + FFA, cholesterol + free fatty acid; TG, triglyceride; CE, cholesterol ester.

matographed in the two-dimensional system described above and the distribution of P^{32} in the phospholipids was studied by autoradiography (13).

Radioactivity Determination

Samples of the various fractions from the rat experiments (C^{14} and P^{32}) were counted in methanolic Instagel scintillation fluid (Methanol: Instagel [Packard Instrument Co. Inc., Downers Grove, Ill. 60515] 1:3 v/v). This system was also used for determining the radioactivity in various lipid classes from thin layer chromatography by scraping the gel from the area and counting directly in scintillator. For colorless samples, chemical quenching was corrected by the channels-ratio method. For color-quenched samples (C^{14} only) internal standard (U-C^{14}-toluene [Packard]) was added, and the samples recounted to correct for this effect.

In the goat experiment (P^{32}) the results were not corrected for quenching and are expressed as counts/min.

To compensate for the decay of P^{32} all samples were either counted at the same time (goat experiment) or counted in conjunction with a P^{32} standard solution, and the results corrected to equivalent activity at the time of injection (rat experiments).

Hydrolysis Studies

In some instances lipids from the C^{14}-experiment were hydrolyzed by incubation in ethanolic KOH (4N KOH in 30% ethanol) at 80 C for 1 hr. After acidification with c.HCl the saponifiable material (fatty acids) was extracted with 3 x 5 volume petroleum ether. After evaporation of the solvent the fatty acids were counted in methanolic Instagel.

The aqueous residues (nonsaponifiable material) were counted whole in 5 volumes of methanolic Instagel, radioactivity due to K^{40} in the medium (from the ethanolic KOH used for saponification) being corrected by using the appropriate blanks.

Other Determinations

Phosphorus, total, inorganic and phospholipid, was assayed either by the method of Fiske and Subbarow (15) or that of Rouser et al. (16).

RESULTS AND DISCUSSION

Metabolism of Uniformly-labelled C^{14}-Phosphatidyl Choline by Lactating Rat Mammary Gland

Uniformly labeled phosphatidyl choline was administered by a dietary route into the serum of a lactating rat, and the radioactivity recovered in the serum and milk examined by the methods described above.

Practically all of the administered radioactivity was absorbed by the animal (total fecal activity 1%). Approximately 16% of the administered isotope was secreted into the milk over the 4 days of the experiment. At sacrifice the liver and mammary glands contained 0.05% and 0.15% of the administered dose respectively. The location of the rest of the activity was not determined.

The results (Fig. 1, Table I) show that the cream fraction contained the majority (93%) of the milk radioactivity, mainly in the form of triglyceride (94%). The activity in the skim-milk fraction also was mainly as triglyceride (92%). Analysis of these lipids by hydrolysis (see Materials and Methods) showed that the fatty acid part of the triglycerides contained 98% and 93% of the activity respectively. The origin of the radioactive fatty acids on these triglycerides is presumably the plasma lipids. Since all the serum lipid classes were radioactive (Table I) we cannot determine from this datum which lipid fraction of the serum was utilized by the mammary gland in this experiment.

Very little activity was recovered in the phospholipid fractions of the milk (Table I).

Analysis of this fraction from cream by hydrolysis showed all of the activity associated with the acyl residues on the lipid. In contrast the activity in the skim-milk phospholipid was distributed between the acyl (45%) and non-saponifiable (55%) portions of the molecule.

Bearing in mind that the original isotope (uniformly-labeled, acyl groups mainly C16 and C18 species) contained only 19-20% of its activity in the "backbone" of the phospholipid, the total amount of milk phospholipid which could have been derived from this portion of the administered isotope (by way of serum phospholipids) can be calculated. The total radioactivity associated with the skim-milk phospholipid over the experimental period was 1% x 2.2% of the original dose (from the text and Table I). Of this activity 55% was associated with the "backbone" of the phospholipid (see text). The contribution of the "backbone" of the dietary phospholipid to milk phospholipid was therefore approximately 0.05%. This demonstrates that the lactating rat mammary gland does not utilize pre-formed exogenous (serum) phospholipids to supplement its supply of phospholipid for milk production.

Metabolism of P^{32}-Phosphate by Lactating Rat Mammary Gland

Two experiments were performed in which P^{32} (as phosphate was injected intravenously into lactating rats, and the radioactivity in blood and milk determined at various times after injection. The results from one experiment are presented in Figure 2. Similar data were obtained in the second experiment (not shown).

In both experiments the administered isotope was rapidly taken up by the mammary gland and secreted into milk. Using the factor of 26 ml/day for milk production (8), then during the experimental period 32% (expt. 1) and 40% (expt. 2) of the administered radioactivity was recovered in the milk.

Most of the radioactivity in the milk was in the form of inorganic P^{32}-phosphate. However some radioactivity was detected in milk phospholipids: 1.9% and 2.5% of the total milk radioactivity in experiments 1 and 2 respectively. The maxima for radioactivity in milk phospholipid and total milk phosphorus occurred at approximately the same time (6 hr) after injection (Fig. 2).

If the inorganic phosphate in milk and the phosphorus necessary for milk phospholipid synthesis were provided from a single pool of phosphate in the tissue, then one would expect that the ratio of the activity in each fraction, i.e., 1.9% and 2.5% as above, would be similar

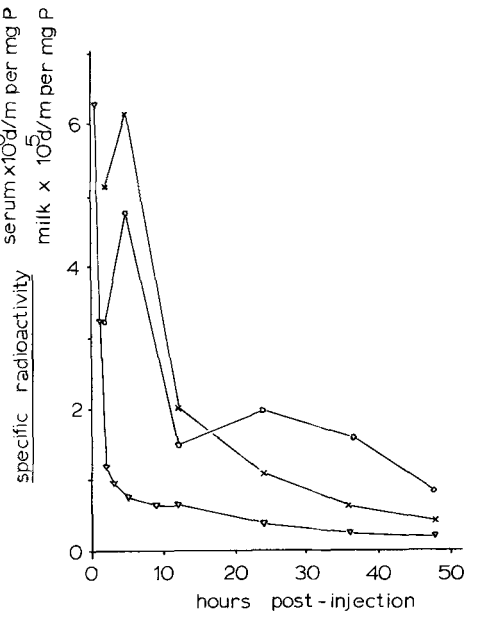

FIG. 2. Serum and milk radioactivity in the rat after P^{32}-phosphate injection. The rat received 25 μc sodium phosphate-P^{32} in saline (0.2 ml) by intravenous injection (caudal vein). Key: -x-, specific activity of non-lipid-P^{32} in milk; -○-, specific activity of lipid-P^{32} in milk; -▽-, specific activity of inorganic P^{32} in serum.

to the ratios of the amounts of phosphorus in each fraction. Values were determined therefore for inorganic phosphorus (2.1 mg/ml) and phospholipid phosphorus (0.04 mg/ml) in rat milk. The mass ratio of phospholipid phosphorus to total phosphorus in rat milk is therefore 1.9%, a value in close agreement to that obtained for the distribution of radioactivity.

Since serum phospholipids (or at least the "backbone" of the molecule) are not utilized to any great extent by the lactating rat mammary gland (demonstrated in Metabolism of Uniformly-labelled . . ., above) for milk phospholipid, these results on the incorporation of inorganic P^{32} into milk phospholipids show that milk phospholipids must be derived mainly (if not totally) by de novo synthesis from inorganic phosphate within the mammary gland.

Metabolism of P^{32}-Phosphate by Lactating Goat Mammary Gland

A lactating goat was injected intravenously with 200 μc inorganic phosphate-P^{32}. Milk and blood samples were collected and fractionated as described (see Materials and Methods).

As shown in Figure 3, in the first 12 hr of

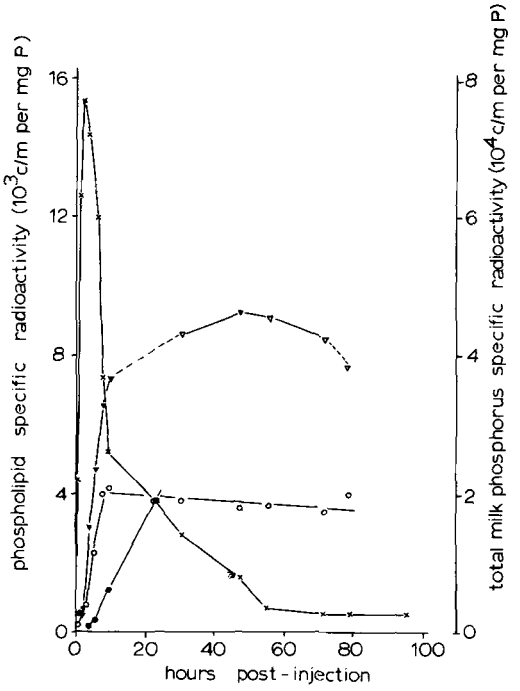

FIG. 3. Serum and milk specific radioactivity in the goat after P^{32}-phosphate injection. The goat received 200 μc orthophosphate-P^{32} in 4 ml of diluted phosphate buffer (pH 7) by intravenous injection (jugular vein). Key: -x-, total P^{32} in milk; -o-, skim-milk lipid P^{32}, -▽-, cream lipid P^{32}; -●-, serum lipid P^{32}.

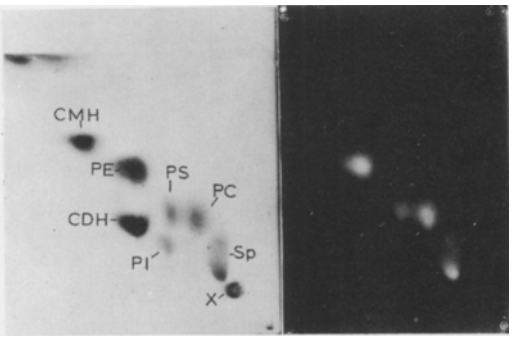

FIG. 4. Autoradiography of goat milk P^{32} phospholipids. Left: Two-dimensional thin layer chromatogram of polar lipids from milk fat globules 31 hr following intravenous injection of P^{32} (phosphate) into a goat. Right: Autoradiogram of the chromatogram. *Abbreviations:* CMH, cerebroside monohexoside; CDH, cerebroside dihexoside; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol Sp, sphingomyelin; X, lactose. Essentially identical chromatographic and autoradiographic data (not shown) were obtained for the skim-milk polar lipids.

the experiment the major form of milk radioactivity was as inorganic phosphate. However after the 12th hr, progressively more of the total milk radioactivity was associated with the skim-milk and cream phospholipids. Autoradiography of samples of cream (Fig. 4) and skim-milk phospholipids from the 31-hr milk indicated that all of the phospholipids normally present in goat milk were synthesized by the goat mammary gland from inorganic phosphate.

By extrapolation of the specific activity results for milk phospholipids past 96 hr, it was estimated that 2.1% of the total milk radioactivity would have been associated with the phospholipid fractions. Since total milk phosphorus is 685 μg/ml (17) and total milk phospholipid phosphorus is 14.7 μg/ml (10), then the corresponding mass ratio (phospholipid phosphorus-total phosphorus) is 2.1%, a value in excellent agreement with the ratio of radioactivity.

We can assume therefore that as with the lactating rat, a single pool of phosphate in the mammary gland is responsible both for supplying inorganic milk phosphate and for supplying the phosphate necessary for milk phospholipid synthesis. Since the specific activity of serum phospholipid is at all times lower than that of cream phospholipid, and for the first 15-20 hr lower than that of the skim-milk phospholipid, it is extremely unlikely for the milk phospholipid to be derived to any great extent from serum phospholipids transported across the mammary gland.

From the positioning of the maximum activities of total milk phosphorus (1.5-6 hr) and milk phospholipid phosphorus (8-80 hr) we must assume that in the goat (but not in the rat, see Metabolism of P^{32}-phosphate . . ., above), phospholipid synthesized within the mammary gland from inorganic phosphate is incorporated into a phospholipid pool within the tissue, and that the delay in buildup of milk phospholipid radioactivity (and slow turnover time) is caused by a gradual turnover of this phospholipid pool within the tissue.

The results from this experiment demonstrate that as with the lactating rat (Metabolism of Uniformly-labelled . . . and Metabolism of P^{32}-Phosphate . . ., above) the lactating goat mammary gland does not utilize circulating pre-formed serum phospholipids for milk production. Instead the gland utilizes a single pool of phosphate within the tissue both for the secretion of inorganic phosphate into the milk and for supplying the phosphorus necessary for milk phospholipid synthesis within the mammary gland.

ACKNOWLEDGMENT

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[Received April 12, 1971]

SHORT COMMUNICATIONS

The Lipids of the Oriental Hornet, *Vespa orientalis* F.

ABSTRACT

Analysis of the hornet's hemolymph revealed the presence of C_{16} and C_{18} fatty acids (70%), which were accompanied by minor quantities (ranging from 0.1% to 0.6%) of the following acids: $C_{10:0}$, $C_{11:0}$, $C_{12:0}$, $C_{13:0}$, $C_{14:0}$, $C_{15:0}$, $C_{16:0}$, and $C_{17:0}$. The hemolymph of the queen larvae contained more $C_{18:1}$ than the hemolymph of the worker larvae, and the percentage of $C_{16:1}$ was higher in the fat body and the midgut than in the hemolymph. The significance of these results is discussed.

Many studies concerning hornets and wasps have emphasized the fact that the larvae of all casts and of both sexes serve, inter alia, as a storehouse for food leavings for all members of the family (1-3). The aim of our work was to find out how these food leavings are kept within the larval body, their location and concentration, as well as the qualitative differences of the lipid constituents, mainly the fatty acids, of larvae of different ages and sexes (J. Ishay and R. Ikan, in preparation).

TABLE I

Lipids Content in Different Tissues of the Oriental Hornet

Source of lipids	Percentage of lipids in dry material
Worker larvae[a]	
Hemolymph	0.4
Midgut	0.2
Fat body	18.6
Total percentage of lipids in the whole body	28.2
Queen larvae[b]	
Hemolymph	0.9
Midgut	11.4
Fat body	20.1
Total percentage of lipids in the whole body	32.4
Queens pupae hemolymph	24.9

[a]Mean value of 200 worker larvae collected during the month of July.

[b]Mean value of 200 queen larvae collected during the month of October.

Lyophyllized hemolymph, fat body and midgut were extracted with chloroform-ether 1:1. The extracts were filtered and concentrated under reduced pressure, yielding viscous residues.

Each of the above extracts was applied on silica gel GF-254 plates (20 x 20 cm; 0.25 and 0.75 mm thick), and the two plates were developed with petrolether-ether-acetic acid (20:4:1) in the same chromatographic chamber. Octadecenoic and palmitic acid and tripalmitin were used as reference compounds. Drying of the plates and spraying of the thin one (0.25 mm) only with 50% sulfuric acid and subsequent charring at 150 C revealed the constituents as black spots. The bands (on the 0.75 mm plates) corresponding to the R_f values of the free fatty acids (FFA) and triglycerides as revealed by the thin plate were scraped off the plates and extracted with chloroform-ether (1:1).

The triglycerides (after removal of solvents) were refluxed for 1 hr with a methanolic solution of potassium hydroxide (5%) and a few drops of water. The solvents were removed in vacuo, water was added, and the solution was extracted with ether. The aqueous phase was treated with 1 N hydrochloric acid and extracted thoroughly with ether. The combined ethereal extracts were dried and the solvent evaporated.

The mixtures of fatty acids (both FFA and BFA) were methylated with diazomethane in ether. The methyl esters were analyzed on a Packard Model 7400 gas chromatograph using: (a) a coiled glass column, 2 m x 6 mm, packed with 1.5% (by weight) of SE-30, coated on Chromosorb W, 80-100 mesh, the column temperature being 190 C; (b) a similar column filled with DEGS 10% on Chromosorb W, column temperature 160 C. The gas flow (nitrogen) was 35 ml/min. The chain length and degree of unsaturation of the compounds was established by comparison of the retention times with those of the methyl esters of pure fatty acids, and by co-injection with these standards.

The weight of lipids found in hemolymph, fat body and midgut are summarized in Table I. The percentage of the extractable lipids of the hemolymph and midgut and fat body of the

TABLE II

Percent of Free and Bound Fatty Acids in the Various Tissue of the Oriental Hornet

	Hemolymph of:					Fat body		Mid gut	
	Worker larvae	Queens larvae		Queens pupae		Worker larvae		Worker larvae	Queens larvae
Acid	FFA	FFA	BFA	FFA	BFA	FFA	BFA	FFA	FFA
10:0	0.1	0.1	0.1	0.1	0.2	0.4	1.1	0.4	1.0
11:0	0.1	0.3	0.3	0.1	0.1	---	---	---	---
12:0	1.5	1.8	1.2	0.9	2.8	1.4	3.0	0.7	0.5
13:0	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
14:0	7.8	6.4	5.8	4.0	8.2	8.4	9.5	4.4	2.7
15:0	0.3	0.2	0.3	0.1	0.1	0.1	0.1	0.1	0.1
16:0	43.6	36.0	36.8	27.5	27.0	24.6	26.2	26.9	25.0
16:1	0.3	0.6	0.4	1.8	1.5	3.1	2.9	4.5	2.5
17:0	0.3	0.5	0.3	0.1	0.5	0.1	0.1	0.1	0.1
18:0	13.0	11.2	12.6	11.4	6.9	11.0	7.4	23.5	10.5
18:1	31.0	39.4	40.3	48.2	51.2	49.4	47.6	33.6	47.9
18:2	1.6	1.9	1.5	5.1	2.3	1.1	1.7	5.0	8.7

queen larvae is higher than of the worker larvae. The high lipid concentration of the midgut of both workers and queens (Tables I and II) indicates that the midgut may function not only as a digestive tract but as storage space as well. A higher concentration of lipids was found in the larvae of queens than in the larvae of workers. A plausible explanation is that since larvae of queens hibernate from autumn to spring (4,5) they need more fat. Workers, on the other hand, do not hibernate but live for not more than 45 to 60 days (during the hot season of the year) and receive nutrition during their lives as imagines.

Vespa orientalis is a carnivorous insect (6), the workers feeding predominantly on honeybees. They also collect carbohydrates from flowers having superficial nectaries. A significant portion of the lipids brought to the nest may also be in the form of butterfly-caterpillars, bees and flies, in which the major fatty acids are: $C_{16:0}$, $C_{16:1}$, $C_{18:0}$ and $C_{18:1}$ (7). The only other published data on species close to the Oriental hornet is on imagines (not larvae) of *Vespa crabro* (8) and honeybees (9).

In our future research work, we intend to bring some data on the alterations in lipids at different developmental stages of the larvae.

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[Revised manuscript received June 18, 1971]

On the Origin of Oil Droplets in Maturing Castor Bean Seeds, *Ricinus communis*

ABSTRACT

Fat droplets isolated from maturing castor bean seeds contain high concentrations of fatty acid synthetase and triglyceride synthesizing enzymes. These activities are not due to contamination by

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18:0	13.0	11.2	12.6	11.4	6.9	11.0	7.4	23.5	10.5
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ABSTRACT

Fat droplets isolated from maturing castor bean seeds contain high concentrations of fatty acid synthetase and triglyceride synthesizing enzymes. These activities are not due to contamination by

TABLE I

Lipid Synthesis by Castor Bean Fractions[a]

Fraction	Fatty acids		Triglyceride
	^{14}C-Acetate	^{14}C-Mal. CoA	^{14}C-Oleyl CoA
Homogenate	1.00	1.00	1.00
1,000-g pellet	0.60	0.55	---
10,000-g pellet	0.50	0.30	0.60
100,000-g pellet	0.25	1.10	0.55
100,000-g sup.	0.00	1.30	0.00
Fat fraction	4.80	3.20	1.80

[a]Results are expressed as Relative Specific Activity of homogenate in terms of protein. Details of the reaction mixture are described in References 6 and 7.

other organelles and account for at least 80% of the total lipid synthesis. Electron microscopy of the isolated oil droplets and the seed tissue in vivo revealed particulate containing vacuole-like inclusions which, it is suggested, are the site of lipid synthesis and which form the original locus of the droplet.

Seeds containing large amounts of lipids have as much as 70% of their dry weight in the form of triglycerides stored as oil globules in the cell. Under the electron microscope these globules appear as uniform bodies with no internal structures or enclosing membranes. They cannot be equated with spherosomes which are reported as small membrane bound bodies containing osmiophilic material (1). Some workers believe spherosomes develop from fragments of endoplasmic reticulum (2,3). Sorokin (1) differentiates quite clearly between the oil droplet and the spherosome, and denies any developmental connection between the two.

Formation of the oil droplet calls for the flow of substrates and cofactors to a specific site where the synthesis and accumulation of lipids as triglycerides could occur. The site would need to possess (a) a high concentration of the fatty acid synthetase enzymes using malonyl CoA for the de novo formation of long chain fatty acids, and (b) the enzymes necessary for the further esterification of the fatty acids to triglycerides.

A typical high lipid containing seed is *Ricinus communis* (castor bean). More than 90% of its esterified fatty acids is ricinoleic acid, D-(+)12-hydroxy-cis-9-octadeconoic acid (4), which is synthesized from oleyl CoA by a mixed function oxidase (5). The maturing seed was chosen as a model for a plausible explanation of oil droplet biogenesis.

The maturing (24-40 days after flowering) seed was subjected to subcellular fractionation by differential and Ficoll density gradient

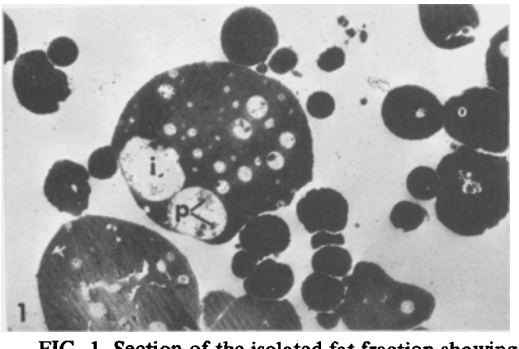

FIG. 1. Section of the isolated fat fraction showing fat globules with vacuole-like inclusions containing particulates: i, inclusion; p, particulates (x 3,590).

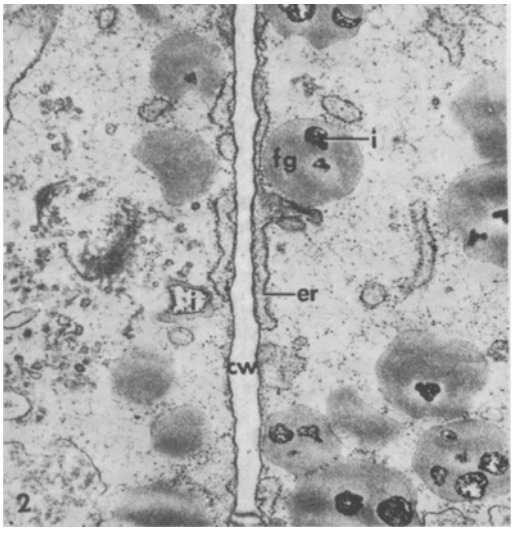

FIG. 2. Portions of two endosperm cells showing profiles of developing fat globules with vacuole-like inclusions containing particulates: fg, fat globule; i, inclusion; er, endoplasmic reticulum; cw, cell wall (x 21,890).

centrifugation. The fractions were incubated with 1-^{14}C-acetate and added cofactors (CoA, bicarbonate, NADH, NADPH, $MnCl_2$, ATP and acyl carrier protein), and the fatty acids produced were analyzed by gas liquid chromatography as previously described (6). The fat droplets that were isolated had the highest specific activity in terms of protein (Table I), and accounted for more than 80% of the total fatty acid synthesis. Extensive washing of the oil droplets, which removed all measurable marker enzyme activities (such as succinate dehydrogenase, glucose-6-phosphatase, and 5'nucleotidase), failed to reduce fatty acid synthesis. Only complete defatting of the fat fraction with acetone at -20 C released soluble fatty acid synthetase. Mild dispersion of the oil droplet lipid with 0.1% Triton X-100 or Lubrol allowed the fatty acid synthesizing enzymes to be sedimented in a 105,000 g x 60 min spin. In addition to acetate, malonyl CoA was also rapidly utilized by the fat fraction in fatty acid synthesis (Table I). Thus the first requirement for oil droplet biogenesis, i.e., a high level of the fatty acid synthesizing enzymes at a specific site, was fulfilled.

Triglyceride synthesis was studied in subcellular fractions by following the incorporation of 1-^{14}C-oleyl CoA in the presence of α-glycerophosphate (7,8). The specific activity of the fat fraction was double that of the homogenate and accounted for the bulk of the total recovered activity. Association of membrane fragments with the outside of the oil droplet was excluded by centrifuging the other organelles in an artificial emulsion of olive oil. The resulting floating layer did not synthesize triglycerides. Therefore the native oil droplets also contained the enzymes necessary for the synthesis of triglycerides.

After demonstrating that the forming oil droplet can synthesize its own lipid and is the primary site of such synthesis in the maturing castor bean, we examined the isolated fat fraction by electron microscopy using a low viscosity epoxy resin for embedding (Fig. 1) (9). The fraction is homogeneous and consists almost entirely of osmiophilic droplets of various sizes, 0.1-3.5 μm diameter. The particulate containing, vacuole-like inclusions within the droplets were of considerable interest. To determine if such inclusions occur in vivo, endosperm tissue at progressive stages of development was also examined by electron microscopy. At 37 days after anthesis, vacuole-like inclusions (0.1-0.3 μm in diameter) were evident in the oil droplets of endosperm cells (Fig. 2). The inclusions also contained an osmiophilic particulate phase indicating that the inclusions

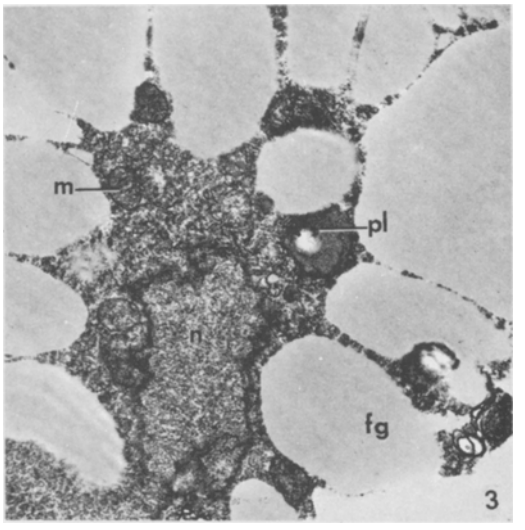

FIG. 3. Portion of a mature endosperm cell showing fat globules lacking vacuole-like inclusions: fg, fat globule; n, nucleus; m, mitochondrion; pl, plastid (x 11,090).

observed in the oil droplets of the isolated fat fraction were not artifacts of the isolation process. We believe that these inclusions are an important site of lipid synthesis in the maturing castor bean endosperm.

Since the oil droplet is in a constant state of flux and agitation (consider the variable shapes of the droplets and of the inclusions Figure 2, and that the inclusions may contact the peripheral interface of the droplet), presumably both substrate and cofactors can, by proximity to the globule interface, become available to the internal enzymes. The fact that the inclusions are discrete regions within the oil droplet is indicative of their more hydrophilic nature in an otherwise lipophilic matrix, and is one basis for considering them the site of hydrophilic enzymes. When the lipid globule is larger and more stable the opportunity for interchange decreases; and for this reason, and perhaps others, fat synthesis ceases. In mature seeds acetate incorporation into lipid is at a very low level (10), and inclusions are no longer present within the fat globule (Fig. 3).

It is significant that the fat droplets lack a peripheral membrane but have a very thin, contrasted and sharply defined interface with the ground substance of the cytoplasm. This is at variance with the assumption that a true membrane surrounds the fat droplet (2). The inclusions within the droplet also lack limiting membranes.

In summary, we have shown that the oil droplets can account for at least 80% of the

total fatty acid and triglyceride synthesis in the maturing castor bean. The location of the synthesizing enzymes in inclusions within the oil droplets suggests a highly specialized site designed for oil droplet biogenesis with the full complement of enzymes necessary for the synthesis of triglycerides from acetate and glycerophosphate. We propose that oil droplets originate in the maturing castor bean seed around a cluster of enzymes in the ground substance of the cell and involve both de novo fatty acid biosynthesis and triglyceride formation. These systems then catalyze the build-up of triglycerides from suitable substances leading to the formation of the fully mature oil droplets (Fig. 3).

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[Received August 13, 1971]

The Structures of Triglycerides From Atherosclerotic Plaques and Other Human Tissues

ABSTRACT

Stereospecific analysis of triglycerides isolated from human liver showed that these were much more asymmetric than the triglycerides from human adipose tissue. Heart muscle triglycerides were similar in structure to adipose tissue triglycerides. The triglycerides from aortic plaques were intermediate in structure between triglycerides of liver and adipose tissue.

It has recently been shown that the triglycerides isolated from the livers of the pig (1), sheep (2), chicken and rabbit (Christie and Moore, unpublished data) differ considerably in structure from those in the adipose tissue of these species. We now report structural analyses of triglycerides isolated from human liver, adipose tissue, heart muscle and aortic plaques.

Tissues were obtained from three patients in the age range of 60-70 years who had died as a consequence of cerebrovascular accidents and who at autopsy were found to have severe atherosclerotic lesions in the aorta of grade III severity according to the classification of Boettcher et al. (3). The plaques were dissected from the aortas and corresponding tissues from each of the patients were pooled before analysis. The lipids were extracted from the tissues with chloroform-methanol 2:1 and the triglycerides were obtained by preparative thin layer chromatography (1). The procedure for stereospecific analysis of triglycerides, i.e., for determining the composition of fatty acids esterified in positions 1,2 and 3 of the glycerol moeity, has been described elsewhere (1,4). The results of the analyses are listed in Table 1.

Human liver triglycerides were highly asymmetric and contained more than 60% 16:0 and 11% 18:0 in position 1, the remainder being largely monoenoic components. Position 2, on the other hand, contained only 12% saturated acids and was occupied largely by 18:1 and 18:2. Position 3 contained somewhat more 16:0 and 18:0 (23%) than position 2 but the principal components were again 18:1 and 18:2. The small amount of polyunsaturated fatty acids were found mainly in position 2.

The adipose tissue triglycerides were similar

total fatty acid and triglyceride synthesis in the maturing castor bean. The location of the synthesizing enzymes in inclusions within the oil droplets suggests a highly specialized site designed for oil droplet biogenesis with the full complement of enzymes necessary for the synthesis of triglycerides from acetate and glycerophosphate. We propose that oil droplets originate in the maturing castor bean seed around a cluster of enzymes in the ground substance of the cell and involve both de novo fatty acid biosynthesis and triglyceride formation. These systems then catalyze the build-up of triglycerides from suitable substances leading to the formation of the fully mature oil droplets (Fig. 3).

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It has recently been shown that the triglycerides isolated from the livers of the pig (1), sheep (2), chicken and rabbit (Christie and Moore, unpublished data) differ considerably in structure from those in the adipose tissue of these species. We now report structural analyses of triglycerides isolated from human liver, adipose tissue, heart muscle and aortic plaques.

Tissues were obtained from three patients in the age range of 60-70 years who had died as a consequence of cerebrovascular accidents and who at autopsy were found to have severe atherosclerotic lesions in the aorta of grade III severity according to the classification of Boettcher et al. (3). The plaques were dissected from the aortas and corresponding tissues from each of the patients were pooled before analysis. The lipids were extracted from the tissues with chloroform-methanol 2:1 and the triglycerides were obtained by preparative thin layer chromatography (1). The procedure for stereospecific analysis of triglycerides, i.e., for determining the composition of fatty acids esterified in positions 1,2 and 3 of the glycerol moeity, has been described elsewhere (1,4). The results of the analyses are listed in Table 1.

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The adipose tissue triglycerides were similar

in structure to those analyzed by Brockerhoff (5). These were still asymmetric although the differences in fatty acid composition between positions 1 and 3 were not as marked as in the liver triglycerides. There were much higher proportions of 14:0 and 16:1 in the adipose tissue triglycerides than in the liver triglycerides and these acids were in greatest concentration in position 2. Heart muscle triglycerides were virtually indistinguishable from adipose tissue triglycerides. This was also true of other species examined (1,2) (Christie and Moore, unpublished data).

The triglycerides of aortic plaques were intermediate in structure between those of liver and adipose tissue. There was a high proportion of 16:0 (54%) in position 1 together with 11% 18:0. Position 2 contained somewhat more 16:0 (15%) than was found in this position in the triglycerides of the other tissues although unsaturated acids again predominated. Position 3 contained only 17% in all of 16:0 and 18:0, and longer chain unsaturated acids predominated in this position also. Significant amounts of 14:0 and 16:0 were also found, although not as much as in adipose tissue triglycerides, and these were in greatest concentration in position 2. Small amounts of long chain polyunsaturated fatty acids were found in positions 2 and 3. These results directly contradict those of Caley et al. (6) who reported that saturated fatty acids occurred principally in position 3 of the triglycerides of aortic plaques.

The differences in structure between liver and adipose tissue triglycerides probably arise from different mechanisms of biosynthesis in these tissues. In adipose tissue, triglyceride synthesis must occur largely by the α-glycerophosphate pathway (7,8) via diglyceride intermediates although there may be a contribution from the monoglyceride pathway (9). In liver, on the other hand, there is much more rapid turnover of lipid classes, and the diglyceride intermediates for triglyceride biosynthesis are produced to a large extent by dephosphorylation, deacylation and resynthesis of other lipids (10). However there is considerable uncertainty about the origin of triglycerides in aortic plaques.

TABLE I

Stereospecific Analysis of Triglycerides From Human Tissues

Tissue	Position	Fatty acid composition														
		14:0	14:1	15:0	16:0	16:1	17:0	17:1	18:0	18:1	18:2	18:3 + 20:0	20:1	20:2	20:3	20:4	20:5
Liver	TG[a]	1.1	---	0.2	28.2	2.5	0.6	---	7.4	50.5	7.1	0.4	0.5	---	0.2	1.3	---
	1	1.1	---	0.2	60.8	3.2	1.2	---	11.5	18.1	2.7	0.1	0.6	---	---	0.5	---
	2	1.4	---	0.2	9.1	3.3	0.6	---	2.7	64.0	14.5	1.0	0.2	---	0.6	2.4	---
	3	0.8	---	0.2	14.7	1.0	---	---	8.0	69.4	4.1	0.1	0.7	---	---	1.0	---
Heart muscle	TG[a]	4.6	0.6	0.3	26.4	8.6	0.4	0.3	6.1	45.6	5.5	0.7	0.9	---	---	---	---
	1	4.4	0.2	0.6	48.3	4.4	---	0.4	11.0	24.4	3.8	1.5	1.0	---	---	---	---
	2	6.0	1.4	0.3	10.1	14.5	0.3	0.4	1.6	54.6	10.1	0.7	---	---	---	---	---
	3	3.4	0.2	---	20.8	6.9	0.9	0.1	5.7	57.8	2.6	-0.1	1.7	---	---	---	---
Adipose tissue	TG[a]	4.6	0.7	0.5	23.9	7.2	0.5	0.5	7.5	46.2	6.3	1.0	1.1	---	---	---	---
	1	3.9	0.1	0.7	42.3	3.4	1.0	---	14.7	27.2	3.8	1.9	1.0	---	---	---	---
	2	6.0	1.3	0.4	10.4	12.0	0.2	0.4	2.0	54.7	11.3	1.1	0.2	---	---	---	---
	3	3.9	0.7	0.4	19.0	6.2	0.3	1.1	5.8	56.7	3.8	---	2.1	---	---	---	---
Aortic lesions	TG[a]	3.1	---	---	26.6	5.4	---	---	6.3	44.5	9.7	0.9	1.0	0.3	0.6	1.2	0.4
	1	2.7	---	---	53.8	3.7	---	---	10.9	24.4	3.6	0.3	0.6	---	---	---	---
	2	3.4	---	---	15.0	7.7	---	---	2.1	51.2	15.7	1.2	0.7	0.2	0.6	1.7	0.5
	3	3.2	---	---	11.0	4.8	---	---	5.9	57.9	9.8	1.2	1.7	0.7	1.2	1.9	0.7

[a]TG = triglycerides.

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[Received May 17, 1971]

Fatty Acid Composition of Triglycerides and Phosphoglycerides During Growth in *Glomerella cingulata*

ABSTRACT

The fatty acid composition of triglycerides and phosphoglycerides accumulated at selected ages during the growth of *Glomerella cingulata* was investigated. Glyceride accumulation was taken as mg glyceride/mg N and the nitrogen content of the fungus at the ages investigated was used as an index of growth. The fatty acids produced were identified by comparing their retention times on gas liquid chromatography with that of known standards. The results showed that whereas total glyceride and fatty acid content varied from age to age, the fatty acid composition at the various ages remained the same.

INTRODUCTION

Triglycerides and phosphoglycerides are the major classes of lipids found in fungi. In a study on the lipids of six-day-old conidia of the fungus *Glomerella cingulata*, Jack (1) had shown that triglycerides accounted for more than 95% of the neutral lipids of the fungus. Four of the five classes of triglycerides present in the fungus contained unsaturated fatty acids. The phospholipid fraction consisted of phosphatidyl-ethanolamine, phosphatidylserine, phosphatidylcholine and an unknown phosphatide fraction (1). Both of these glycerides however contained the same major classes of fatty acids: C_{14}, C_{16}, $C_{16}(1=)$, C_{18}, $C_{18}(1=)$, $C_{18}(2=)$, $C_{18}(3=)$. However little is known concerning the fatty acid composition of the glycerides at earlier periods of fungal growth. Since some microorganisms are known to be capable of synthesizing different fatty acids during growth (2) it appeared of interest to study the fatty acid composition of the glycerides at selected ages during growth of *G. cingulata*.

The culture of *G. cingulata* used in this study was obtained originally from Jack Ziffer Pabst Laboratories, Milwaukee, Wisconsin, and was maintained on potato dextrose agar. About 87 x 10^6 spores were used to inoculate 1000 ml of nutrient medium, supplemented with 1 ml micronutrient solution. The compositions of

TABLE I

Quantitative Measurement of Fungal Growth, Total Lipids, Triglycerides and Phosphoglycerides

Days	Growth, (total mg nitrogen)	Total lipids, mg/ml	Triglycerides, mg/ml	Phosphoglycerides, mg/ml
1.5	12.5	2.7	1.3	1.5
2	15.0	3.6	1.8	2.0
3	14.0	3.6	1.8	2.0
4	13.0	2.7	1.0	1.3
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3	14.0	3.6	1.8	2.0
4	13.0	2.7	1.0	1.3
6	15.0	2.6	1.0	1.5

TABLE II

Areas[a] of Methylated Fatty Acids Obtained from a Growing Culture of *Glomerella Cingulata*

	Triglycerides			Phosphoglycerides		
Days	C_{14}	C_{16} group	C_{18} group	C_{14}	C_{16} group	C_{18} group
1.5	11.7	1.8	6.6	1.3	0.6	2.6
2	18.9	1.9	7.6	12.4	2.4	7.6
3	26.4	2.3	9.1	13.5	2.8	8.2
4	18.8	0.8	2.7	0.8	0.4	0.8
6	8.8	0.8	3.0	1.8	0.8	1.3

[a]In cm.

the nutrient medium and the micronutrient solution were as given by Jack (3). The material was incubated with shaking at 22 C and 25 ml of the mycelial sample was taken from the growing culture at 1.5, 2, 3, 4 and 6 days of fungal growth.

The 25 ml sample was mixed with 150 ml chloroform-methanol 1:1 and macerated in the large cup of a Waring blender. Five milliliters of this slurry was taken for nitrogen determination by Nesslerization. Total lipid extraction from the remaining 170 ml was carried out as reported by Jack (3).

The triglycerides and phosphoglycerides were separated from the rest of the lipids and from each other by column chromatography on silicic acid. The glycerides were purified by gel filtration using Sephadex G-25 and the lipids monitored by thin layer chromatography on Silica Gel G. Glyceride quantitation was done by the method of Snyder and Stephens (4). Free fatty acids were obtained from the glycerides by hydrolysis with 0.5 N methanolic KOH and the methyl esters of these fatty acids prepared for gas liquid chromatography by esterification of the fatty acids with boron trifluoride methanol reagent. A Microtek gas chromatograph, Model DSS 161, with a thermo conductivity detector was used. Column dimensions: 6.3 ft x 1/4 in., internal diameter; column packing 5% S.E. 30 on chromosorb-P 80/90 mesh; inlet temperature 210 C, oven temperature 185 C and detector temperature, 200 C; carrier gas Helium, inlet pressure 40 psig, sample size: 50 μl.

The methyl esters were identified by comparison of their retention times to those of known standards, and peak areas were obtained by multiplying the length of the peaks by the width at half length.

RESULTS AND DISCUSSION

In preliminary experiments it was observed that the quantities of phosphoglycerides (PG) and triglycerides (TG) at the various ages differed considerably. This is shown in Table I. Maximal glyceride accumulation occurred somewhat between two and three days of fungal growth, although glyceride accumulation and fungal growth exhibited a rise by six days of age. This was observed in previous studies (5) and may be due to secondary growth in the culture. Table II shows the peak areas of the fatty acid methyl esters obtained from the triglycerides and phosphoglycerides at the ages studied. The same classes of fatty acids–C_{14}, C_{16} group (saturated and unsaturated) C_{18} group (saturated and unsaturated)–were found at all the ages, although the quantities differed from age to age. Thus there was generally maximal fatty acid production between two and three days, and a general rise in fatty acid content at six days.

It is apparent that the triglycerides and phosphoglycerides contained the same major classes of fatty acids at the selected ages studied. It is also apparent that maximal fatty acid production as well as maximal glyceride accumulation occurred between two and three days of fungal growth.

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Early Steps on the in Vivo Incorporation of 1-^{14}C-Linoleic Acid Into Liver Lipids From Normal and Essential Fatty Acid Deficient Rats

ABSTRACT

Essential fatty acid (EFA) deficient rats were injected intraportally with a solution of 1-^{14}C-linoleic acid during a 1 min period. Livers were quickly frozen, pulverized, and the lipids extracted and fractioned by thin layer chromatography. The incorporation of 1-^{14}C-linoleic acid into liver lipids was measured. The results were compared with those previously obtained from normal rats. No significant differences were observed in the total radioactivity recovered from lipid extracts. While the distribution of radioactivity into the 1-2 diacylglycerol fraction remained unchanged in both groups of rats, in the EFA deficient rats the 1-^{14}C-linoleic acid incorporation was actually directed to the phospholipid fractions instead of to the triacylglycerol fractions as was observed in the normal rats.

It is well recognized that essential fatty acids (EFA) play an important role in the structure and function of membranes. The structural and functional alterations observed in EFA deficiency were related to a change in the fatty acid composition of the phospholipids of the membrane (1).

In recent experiments (DeTomas and Mercuri, unpublished) (2) we have studied the early steps of the in vivo incorporation of 1-^{14}C-linoleic acid into liver lipids from normal rats. In these experiments it was found that there was a higher incorporation of radiolinoleic acid into the triacylglycerol fractions than in the phosphatidylcholine fractions.

A similar experiment was performed in order to elucidate if this normal scheme of incorporation became different in the EFA deficient rats.

Weanling male Wistar rats maintained during 90 days on a semisynthetic fat free diet (3) were used. Under ether anesthesia 0.1 ml of labeled solution (4) containing 10 μc 1-^{14}C linoleic acid (Radiochemical Centre, Amersham, England, (52.9 mc/mmole) was injected into the exposed portal vein during a 1 min period. After the injection the livers were quickly frozen with liquid N_2, pulverized and the powder extracted with chloroform-methanol 2:1 (5). The lipids recovered from the extract were fractioned by thin layer chromatography (6,7). The lipid fractions were transesterified from the silica gel (8) and the extracted methyl esters assayed for radioactivity in a Packard Tri-Carb Scintillation Spectrometer. Phospholipid hydrolysis was performed as described earlier (2).

Table I summarizes the distribution of the total radioactivity expressed as percentage of 1-^{14}C-linoleic acid incorporated into the principal lipid fractions. No significant differences were observed in the per cent distribution of 1-^{14}C-linoleic acid incorporated into 1-2 diacyl-

TABLE I

Per Cent of the Total Radioactivity Recovered From the 1-^{14}C-linoleic Acid Incorporated Into Lipid Fractions[a]

Experimental group	Per cent distribution of radioactivity			
	1-2 Diacylglycerol	Triacylglycerol	3-*s*-*n*-Phosphatidyl-choline	3-*sn*-Phosphatidyl-ethanolamine
Normal[b](6)	26.7 ± 3.8 N.S.[d]	38.3 ± 4.1 P <0.001	18.3 ± 3.9 P <0.001	5.0 ± 1.2 N.S.[d]
Normal[c](5)	26.7 ± 3.9 N.S.[d]	35.6 ± 4.4 P <0.001	20.4 ± 2.7 P <0.001	4.0 ± 0.8 P<0.02
Essential fatty acid deficient (5)	19.0 ± 7.2	14.5 ± 3.7	49.9 ± 2.2	8.0 ± 2.5

[a]Numbers in parenthesis indicate the number of animals in each group. Data are the means ± standard deviations of the means. Probability (P) values are related to essential fatty acid deficient group.

[b]De Tomas and Mercuri, unpublished.

[c]Reference 2.

[d]N.S., not significant.

glycerol fractions from normal rats compared with the EFA deficient rats.

Coincidently with a fall in the amount of 1-^{14}C-linoleic acid incorporated into the triacylglycerol fraction from EFA deficient rats, there was a rise in the level of radiolinoleic acid incorporated into the lecithin fraction from this group of rats compared with the normal rats.

If we accept that the fatty acid pattern at the 3 position of triacylglycerol (9-12) is a reflection of the composition of the liver acyl-CoA pool, the decrease in the capacity of EFA deficient animals to incorporate linoleic acid into triglycerides could be due to a defect of linoleyl-CoA in this pool. On the other hand the increase of incorporation of 1-^{14}C-linoleic acid into 3-*sn*-phosphatidylcholine from EFA deficient rats compared with the normal rats could be explained either by an increase in the rate of the novo biosynthesis of lecithin (13,14) or by a rise of incorporation through the acylation-reacylation pathway (15). A similar conclusion could be drawn with regard to the 1-^{14}C-linoleic acid incorporation into 3-*sn*-phosphatidylethanolamine.

In the present experiment the mean value of 1-^{14}C-linoleic acid incorporated into total lipids was 5.95 ± 1.75 nmoles/g liver tissue, resembling the data obtained from normal rats. Despite the low availability of linoleic acid such an incorporation might be explained by the high turnover rate of lecithin observed in EFA deficient rats (9,13). As expected the incorporation of labeled linoleic acid into the lecithin, 96%, and cephalin, 94%, fractions from EFA deficient rats occurs primarily into the 2 position as in the normal rats (2).

In conclusion we can suggest that in the EFA deficient rats the in vivo incorporation of linoleic acid is principally directed to the phospholipid fractions.

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TABLE I

Composition of Pooled Lipids From Human Bronchial Asthmatic Mucus

	Neutral lipids[a]			Phospholipids[a]	
Components	mg	%	Components	mg	%
Neutral lipids	75.5	64.8	Phospholipids	24.6	21.1
Cholesterol esters	3.3	2.8	Phosphatidylethanolamine	5.8	5.0
Triglycerides	45.4	39.0	Unknown	1.7	1.4
Free fatty acids	21.1	18.1	Phosphatidylcholine	12.8	11.0
Cholesterol	3.3	2.8	Lysophosphatidylcholine and sphingomyelin	4.3	3.7
Diglycerides	1.1	1.0			
Monoglycerides	1.4	1.2			

[a]Per cent of total lipid weight.

analyzed for lipids and because it could be obtained, free of contamination, from patients with bronchial asthma, an allergic condition characterized by a greatly increased secretion of mucus. The large quantities of mucus obtainable from such a patient permitted quantitative lipid analysis. Smaller quantities of mucus were obtained from patients with chronic bronchitis uncomplicated by infections, but the small yields of lipid generally permitted only qualitative lipid analyses.

Six daily collections of mucus were obtained from a 51-year-old male patient hospitalized with bronchial asthma. His medication included bronchodilators and synthetic corticosteroids. The mucus samples were cloudy but colorless. Microscopically they appeared to be free of pus. The smaller samples of bronchitis mucus were similar in appearance.

Weighed samples of mucus were homogenized at slow speeds in 20 volumes of chloroform-methanol, 2:1, v/v. The chloroform layer was removed and the aqueous layer was extracted three times with equal volumes of chloroform. Treatment of the combined extracts, analysis by thin layer chromatography (TLC), and chromatography of lipids pooled from two samples of asthmatic mucus on neutral and acid-treated Florisil (Floridin Co.) followed techniques previously described (6,7). Chromatography of the neutral lipids was monitored by TLC and showed adequate separations except for cholesterol, which tailed slightly into the monoglyceride fraction. Free fatty acids (FFA) were adsorbed on an ion exchange resin, IRA 400 (Rohm & Haas) (8), and determined by weight loss of the lipid residue. Phospholipids were separated for fatty acid analysis and phosphorus determinations on 350 μ layers of Silica Gel H (Merck) in chloroform-methanol-water, 68:27:3, v/v/v. Rhodamine 6G was used as indicator and the phospholipids were identified by comparison with the known phospholipids from egg yolk. Phosphorus was determined in each of the fractions by the method of Fiske and Subbarow (9). Following saponification fatty acids were methylated with BF_3-methanol. The FFA on the ion exchange resin were methylated by refluxing the resin in HCl-methanol. The fatty acid methyl esters and hydrocarbon fraction were analyzed by gas liquid chromatography as previously described (7,10). The hydrocarbon fraction, 0.1 mg, had a composition similar to that obtained from a blank analysis and is regarded as a contaminant.

Lipids were found in all of the mucus samples and comprised from 0.067%- 0.157%, w/w. The lipid composition of the asthmatic mucus (Table I) appears to be typical of bronchial mucus, for the lipids from three samples of bronchitic mucus showed the same qualitative composition. Fish cutaneous mucus contains much more lipid than bronchial mucus (approximately 1-2%), but the compositions are similar except that squalene and wax esters were not detected in bronchial mucus. The incomplete data for the lipid analyses from other types of mucus agree with the pattern established in fish, snail and bronchial mucus in that two of the major components, cholesterol and phospholipids, are always present. Thus mucus lipids seem to be characterized by variable amounts of neutral lipids and a more constant group of phospholipids in which phosphatidylcholine is the major component. Such a spectrum of lipids from a water dispersable medium in which there is a considerable amount of protein suggests that they occur as a lipoprotein complex. If this is true the large quantities of carbohydrate in mucus would make the term lipoglycoprotein more appropriate.

An interesting feature of the lipids from asthmatic mucus is the large amount of FFA, comprising 18.1% of the total lipids. This concentration of FFA is similar to that found in fish mucus, 10-23%, and human sebum, 10-20% (6,11). The fungistatic effectiveness of

TABLE II

Fatty Acid Composition of the Major Lipid Components of Human Bronchial Mucus[a,b]

Fatty Acid	Cholesterol esters	Triglycerides	Free fatty acids	Phosphatidylcholine
8:0	—	3.1	—	—
10:0	—	3.8	—	—
12:0	1.2	46.7	1.2	—
14:0	3.0	12.6	2.2	2.8
15:0	Trace	Trace	1.1	—
16:0	17.6	12.7	32.8	45.3
16:1	8.0	0.6	4.0	1.2
18:0	5.1	9.6	20.0	19.2
18:1	26.4	8.0	30.7	22.2
18:2	37.7	1.1	8.0	9.2
18:3	0.9	—	Trace	Trace
20:2	—	1.8	—	—

[a]For lipid composition see Table I.

[b]Data in weight per cent.

FFA in human sebum has been the subject of controversy (12). The occurrence of FFA in mucus from exposed, wet surfaces such as fish epidermis, snail epithelium and bronchial epithelium suggests that the question should be reappraised.

The FFA content of bronchial mucus can vary widely. FFA comprised 18.1% of the pooled asthmatic mucus lipids (Table I), but additional samples of mucus from this patient contained 19.2% and 3.8% FFA. Similarly the lipids from one sample of bronchitis mucus contained 19.6% FFA but another, from a different bronchitis patient, had 71% FFA. Analyses of these lipids by TLC confirmed the results and showed a reciprocal relationship between the FFA and triglyceride concentrations. This suggests that the FFA originate by lipolysis of triglycerides similar to that which occurs in human sebum (11). The FFA content of sebum is increased by longer exposure to the skin surface. Possibly the very low, 3.8%, and high, 71%, FFA contents in bronchial mucus also indicate freshly secreted and old mucus. It should be noted that the lipid containing 3.8% FFA had 50% saturation in the triglycerides, but in the lipid with 18.1% FFA the triglycerides were 88.5% saturated, and at 71% FFA saturation reached 90%. This indicates a slight selective lipolysis of triglycerides containing unsaturated fatty acids.

Many of the triglyceride fractions contained unusually large amounts of short chain fatty acids. In the triglycerides from the lipid having 18.1% FFA, lauric acid comprised 46.7% of the total acids and the C_8 and C_{10} acids amounted to 3.1% and 3.8% (Table II). In a different mucus sample from the same patient, the triglyceride fraction contained 43.8% lauric acid and 9.5% each of the C_8 and C_{10} acids. These concentrations are unusual, for human depot fat contains 1-2% lauric acid and only traces of the shorter acids.

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[Revised manuscript received May 10, 1971]

LETTERS TO THE EDITOR

Sphingosine (4-Sphingenine) Nomenclature

Sir: During 1965 I corresponded with a number of chemists who had a primary interest in the sphingosines, discussing the pros and cons of a new system of naming the sphingosines. Following a discussion with the subcommittee on lipid nomenclature of the IUPAC-IUB, I agreed not to introduce a new system but rather to accept the nomenclature that the committee had developed. The Document for Discussion (The Nomenclature of Lipids) prepared by the subcommittee was published in 1966 and 1967 in a number of journals; thus the nomenclature was made available to the chemists in the field.

In the five years since the publication of the Document for Discussion, my experiences as an editor and as an editor responsible for indexing, and my own reflections have led me to the following conclusions:

1. The sphinganine-based semi-systematic nomenclature should be abandoned because (a) the semi-systematic nomenclature may not conserve space and is sometimes less informative than the formal organic chemistry nomenclature. This is illustrated by the table presented in the recent review article by Karlsson (Lipids 5:878, 1970). (b) The nomenclature in actual use is in fact determined by editorial boards and at least the editorial board of Lipids has not chosen to be firm in requiring the sphinganine system. (c) Authors writing in the field of sphingosine chemistry in the years 1969 and 1970 and publishing in Lipids chose six times out of seven, and in J. Lipid Research four times out of seven, not to use the sphinganine system.

2. Since the fully systematic system of naming organic compounds (J. Amer. Chem. Soc. 82:5545, 1960) can be used to completely describe the various sphingosines, it appears that the need is for a useful trivial nomenclature and short-hand notation. I therefore suggest that a simple modified procedure of Prostenik's be employed for developing easily understandable trivial names. In this procedure sphingosine, dihydrosphingosine, and phytosphingosine will retain the classical definitions. When necessary the carbon chain length of sphingosine, etc., and homologs would be indicated by simply writing the carbon number with a hyphen preceding the name, e.g., 20-sphingosine. The configuration would be presumed to be D-*erythro-* unless indicated otherwise, i.e., D-*threo*, etc. If it were necessary to indicate the position of a double bond different from the 4 double bond in sphingosine, one would employ the short hand representation suggested for fatty acids (Kishimoto and Radin, J. Lipid Res. 5:94, 1964), e.g., 18:1 (8 *trans*)-dihydrosphingosine. Other illustrations of trivial, short hand nomenclature exist (Burton, R.M., in "Lipids and Lipidoses," Edited by G. Schettler, Springer-Verlag, New York, 1967). There are certainly deficiencies in Prostenik's system; for example how would one identify the acetylenic analog of sphingosine? However as a short hand, trivial system Prostenik's is an easy to use, easy to write, easy to speak and for the usual sphingosine homologs and analogs the meaning is immediately apparent. One can always use the formal systematic organic chemical nomenclature whenever precise identification is required.

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[Received July 12, 1971]

Shorthand Notation for Multiple-Branched Fatty Acids

Sir: Whereas it is now common practice to use a shorthand notation compatible with the systematic Geneva nomenclature to indicate the structure of unsaturated fatty acids and certain other substituted acids (Holman, Progr. Chem. Fats 9:3, 1968), no similar notation has been proposed for acids with one or more branches. In describing those acids with several methyl

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If a symbol were used to indicate the length of the branch chain (Me = methyl, Et = ethyl, etc.), a system of notation very similar to that for unsaturated acids could emerge, with numerals to indicate the branch points on the main chain. The following examples will clarify the use of this system: Me18:0 would represent methyloctadecanoic acid where the branch point is unspecified; 2-Me18:0, 2-methyloctadecanoic; 2-Me-9-18:1, 2-methyl-9-octadecenoic; 2,4,6-$Me_3$8:0, 2,4,6-trimethyloctanoic; 2,4-Me_2-6-Et8:0, 2,4-dimethyl-6-ethyloctanoic; and, where the steric configurations are known, 2L, 4D, 6D - $Me_3$8:0 (Odham, Ark. Kemi. 27:251, 1967). The structures of these compounds can be seen immediately, as each abbreviation is also "a direct transliteration from the Geneva nomenclature" (Holman, loc. cit.). The use of subscripts is essential where the positions of substituents are unspecified and even where they seem redundant their insertion makes translation easier. Lower case prefixes, such as me instead of Me, would be easily confused with prefixes already used to indicate functional groups such as epoxy (ep).

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ERRATUM

In the short communication "Pristane and Other Hydrocarbons in Some Freshwater and Marine Fish Oils" by R.G. Ackman, Lipids 6:520 (1971), the oil cited as being from the livers of Pacific (gray) cod *Gadus macrocephalus* has been identified as liver oil from the Pacific dogfish listed by some authorities as *Squalus suckleyi* (Girard), which was formerly sometimes called the "grayfish" as indicated in Table I of the communication. This elasmobranch species is now usually considered to be identical with the Atlantic species *Squalus acanthias* Linnaeus, for which a virtually identical level of 0.0157% pristane in the liver oil has been recorded [L.L. Gershbein and E.J. Singh, JAOCS 46:554 (1969)].

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The following figures were omitted from "The Infrared Spectra and Polymorphism of Long Chain Esters: IV. Some Esters From Tetradecanol, Hexadecanol, Octadecanol, Eicosanol, Docosanol and Dodecanoic, Tetradecanoic, Hexadecanoic, Octadecanoic and Eicosanoic Acid," by Aleby et al., which was published in June *Lipids*.

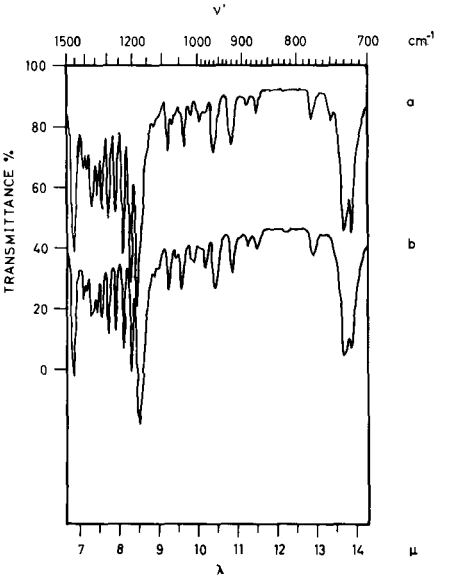

FIG. 3. IR spectra of dodecanoic acid esters. a, Tetradecyl dodecanoate; b, hexadecyl dodecanoate.

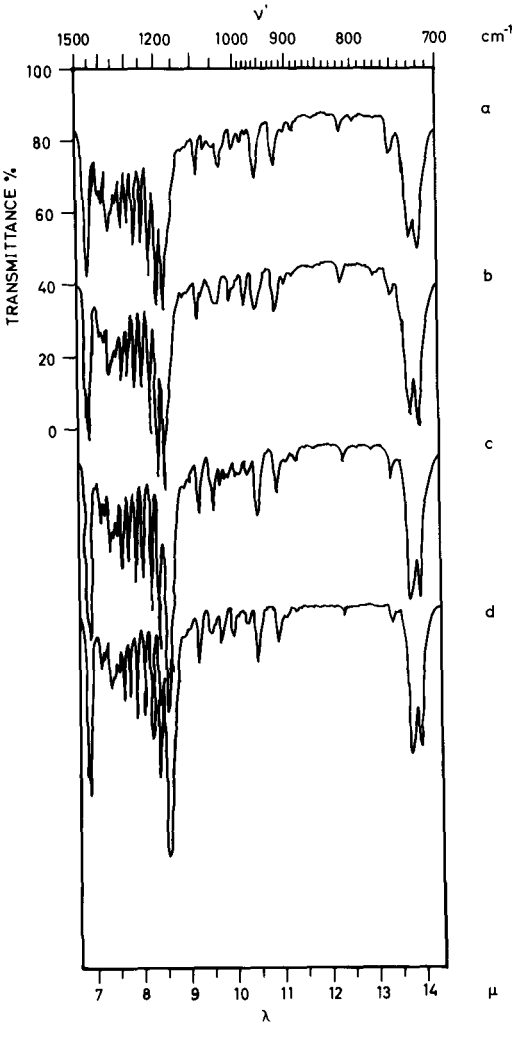

FIG. 4. IR spectra of tetradecanoic acid esters. a, Tetradecyl tetradecanoate; b, hexadecyl tetradecanoate; c, octadecyl tetradecanoate; d, eicosyl tetradecanoate.

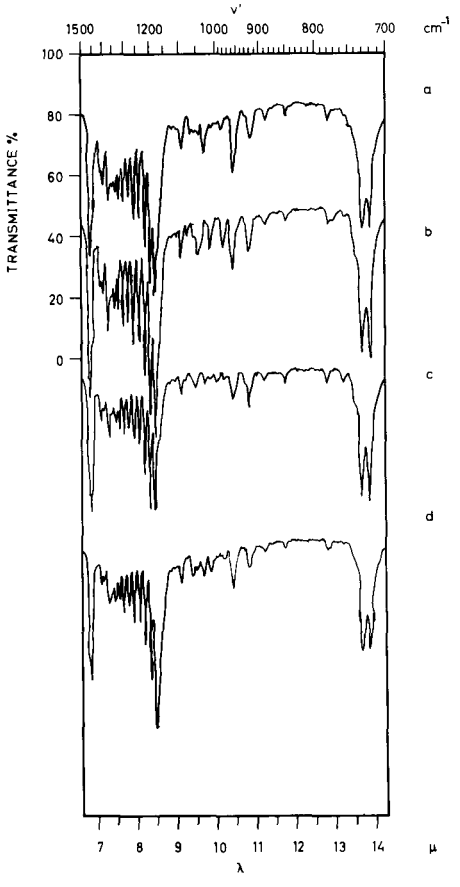

FIG. 5. IR spectra of hexadecanoic acid esters. a, Tetradecyl hexadecanoate; b, hexadecyl hexadecanoate; c, octadecyl hexadecanoate; d, eicosyl hexadecanoate.

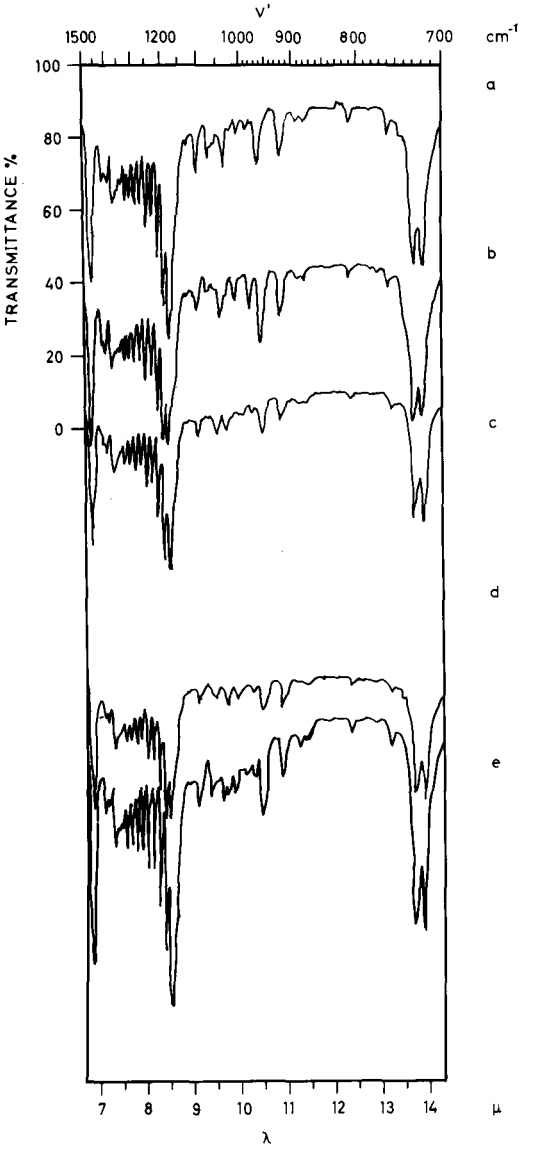

FIG. 6. IR spectra of octadecanoic acid esters. a, Tetradecyl octadecanoate; b, hexadecyl octadecanoate; c, octadecyl octadecanoate; d, eicosyl octadecanoate; e, docosyl octadecanoate.

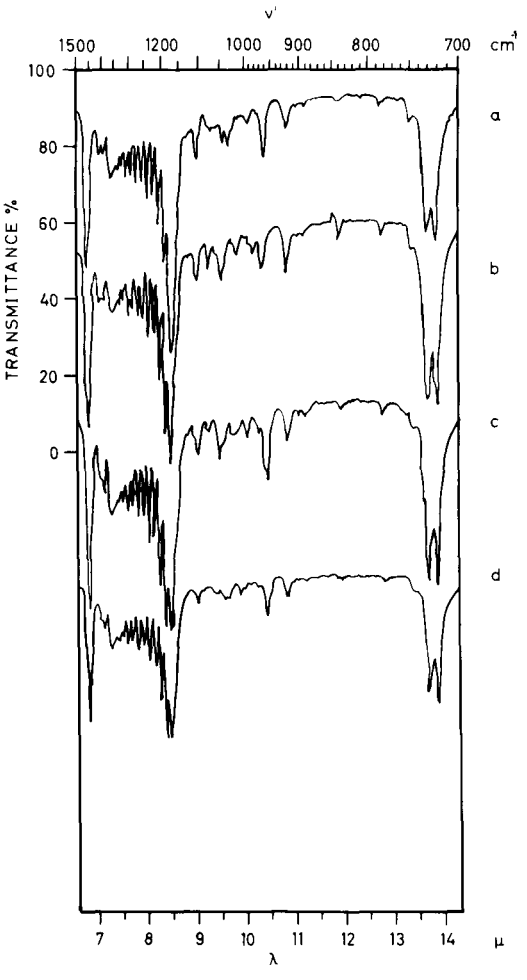

FIG. 7. IR spectra of eicosanoic acid esters. a, Tetradecyl eicosanoate; b, hexadecyl eicosanoate; c, octadecyl eicosanoate; d, eicosyl eicosanoate.

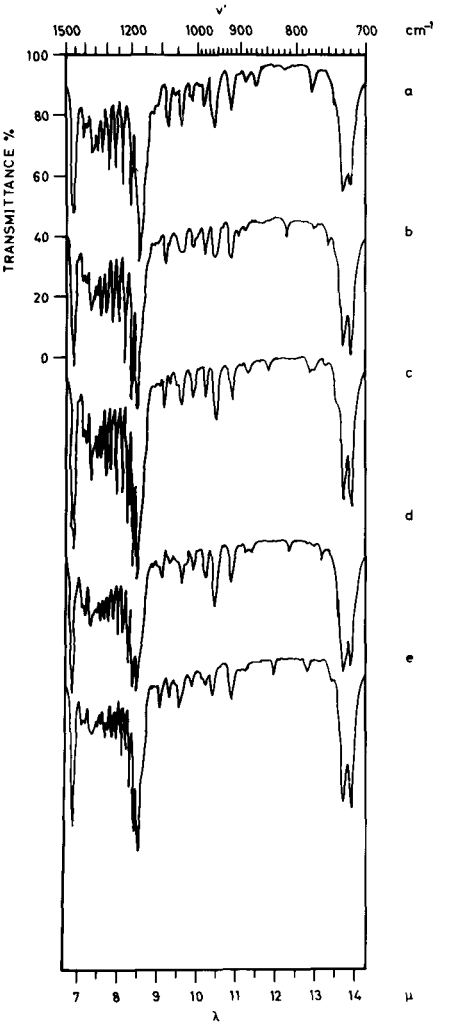

FIG. 8. IR spectra of hexadecyl esters. a, Hexadecyl dodecanoate; b, hexadecyl tetradecanoate; c, hexadecyl hexadecanoate; d, hexadecyl octadecanoate; e, hexadecyl eicosanoate.

On the Phospholipids of *Culex pipiens fatigans*

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ABSTRACT

The lipids of different developmental stages of *Culex pipiens fatigans*, vector of bancroftian filariasis, have been investigated. The phospholipid composition of the developmental stages and of the subcellular fractions of fourth instar larvae of the insects were analyzed. The composition of fatty acids and their positional distribution have also been examined in the major phospholipids of the larvae. The insect eggs contained higher amounts of lipids than larvae suggesting that they were utilized during embryogenesis. Phosphatidyl ethanolamine (PE) and phosphatidyl choline (PC) comprised over 75% of the insect phospholipids. Of these, PE was present in the greatest amounts during all stages of growth and in the subcellular fractions of larvae. An ethanolamine containing sphingolipid was found as a component of the phospholipids of the insects. About 50% of the lipids of the larvae were localized in the cell debris and nuclei fraction which also contained most of the lysolipids of the insects. As in other Diptera 16:0, 16:1 and 18:1 were the major fatty acids present in the insect lipids of which the fatty acid found in greatest amounts was 16:1. Similar to the phospholipids of animal species, saturated fatty acids were predominantly linked to the 1 position of the major phospholipids of the insects while the unsaturated fatty acids were in higher amounts at the 2 position.

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INTRODUCTION

Lipids are known to be of great importance in the metabolism of insects (1). They were reported to be the main source of energy in several insects for embryogenesis (2). Some insects derive their source of energy from lipids during flight (3,4). Buxton (5) observed considerable reduction in fat reserves of hibernating *C. pipiens* suggesting their utilization during hibernation. As part of our detailed study of the lipid metabolism of mosquitoes in relation to public health, the nature of lipids is under investigation in *C. pipiens fatigans*, vector of bancroftian filariasis. In view of the presence of phospholipases observed earlier (6,7) in different developmental stages of the insects, composition of the phospholipids at these stages and their subcellular distribution in fourth instar larvae have been examined and the results are here reported.

MATERIALS AND METHODS

Egg lecithin, 1-acyl glyceryl phosphoryl choline, 1-acyl glyceryl phosphoryl ethanolamine, phosphatidic acid (PA), sphingomyelin and sphingosine were purchased from Biochemicals Unit, Council of Scientific and Industrial Research, Delhi. Phosphatidyl ethanolamine (PE) was isolated from egg lipids by chromatography on columns of silicic acid by stepwise elution with increasing concentrations of methanol in chloroform (7). A chloroform-methanol (17:3) fraction containing PE was collected. Phosphatidyl serine (PS) was isolated from rat brain lipids and was purified by thin layer chromatography. Cardiolipin (C) was isolated from *Mycobacterium 607* as described in an earlier publication (8). All developmental stages of the insects were obtained from large numbers of egg rafts of *Culex* and reared in yeast medium

TABLE I

Lipids of Developmental Stages of *Culex pipiens fatigans*

	Lipids, mg/g wet wt.		
Developmental stage	Total	Phospholipid	Neutral lipid
Egg rafts	105	17.7	87.3
Larvae, fourth instar	34.3	8.6	25.7
Pupae	58.2	8.0	50.2
Adults	78.7	7.9	70.8

TABLE II

Phospholipid Composition of Developmental Stages of *Culex pipiens fatigans*

Developmental stage	Per cent total phospholipids								
	PA[a]	C	PE	PC	SPL	LPE	PI	LPC	PS
Egg rafts	3	4	37	34	—	7	5	3	6
Larvae	—	2	59	19	4	5	4	3	5
Pupae	—	3	50	21	4	7	4	4	5
Adults	—	—	57	20	5	6	2	3	5

[a]Abbreviations: PA, phosphatidic acid; C, cardiolipin; PE, phosphatidyl ethanolamine; PC, phosphatidyl choline; SPL, sphingolipid; LPE, lysophosphatidyl ethanolamine; PI, phosphatidyl inositol; LPC, lysophosphatidyl choline; PS, phosphatidyl serine.

at 25-27 C. The resulting fourth instar larvae, pupae and adults of both sexes collected within a few hours after emergence were used for extraction of lipids. Cobra venom (*Naja naja*) was purchased from Haffkine Institute, Bombay.

Egg rafts lifted by a brush were placed on filter papers, gently blotted to remove moisture and weighed. Larvae and pupae were washed with cold distilled water, blotted briefly and quickly weighed. Adults were collected in a cold test tube, killed by freezing and weighed. The insect material was transferred to a Potter-Elvejhem glass tube and homogenized with 20 volumes of chloroform-methanol (2:1). The homogenate was taken in a conical flask with an additional volume of chloroform-methanol and filtered. The resulting lipid extract was purified by the method of Folch et al. (9) and evaporated on a rotary evaporator under vacuum in a water bath maintained below 50 C. The residue was dissolved in ca. 10 ml chloroform-methanol (2:1) containing 4% water and evaporated to dryness. This procedure was repeated to remove any protein contaminant. The residue was finally taken in suitable fractions of chloroform and preserved at -20 C in nitrogen atmosphere until used.

Subcellular fractions were prepared by centrifugation of a 10% fourth instar larval homogenate in 0.25 M sucrose in VAC-60 preparative ultracentrifuge at 2 C at 1000 g, 13,000 g and 105,000 g for separation of nuclei, mitochondria, microsomes and supernatant respectively. Purity of the fractions was checked by specific enzyme assays (10,11). Using the marker enzymes L-glutamate: NAD oxidoreductase (E.C.1.4.1.2) for mitochondria and D-glucose 6-phosphate phosphohydrolase (E.C. 3.1.3.9) for microsomes, cross contamination between the fractions was found to be ca. 10%. Lipids were isolated from nuclei, mitochondria and microsomes by the above procedure for the whole insects. Lipids of supernatant were extracted by the method of Bligh and Dyer (12).

TABLE III

Lipids of Subcellular Fractions of Fourth Instar Larvae, *Culex pipiens fatigans*

Subcellular fraction	Per cent larval lipid	
	Neutral lipid	Phospholipid
Cell debris and nuclei, 1000 g, 10 min	49	53
Mitochondria, 13,000 g, 20 min	18	29
Microsomes, 105,000 g, 60 min	27	14
Supernatant	6	3

Total lipids were determined by gravimetry. Phospholipids were quantitated by multiplying the phosphorus value estimated by the Bartlett procedure (13) with 25. Neutral lipid content was calculated by difference.

One and two dimensional thin layer chromatography (TLC) on glass plates coated with Silica Gel G (E. Merck) was employed extensively for identification, quantitation and isolation of the lipids. Neutral lipids were separated on TLC using petroleum ether (40-60 C)-ether-acetic acid (90:10:1 and 30:70:1 respectively) as developing solvents. Neutral lipids were located on plates by using authentic markers with dichlorofluorescein spray and iodine exposure. Phospholipids were separated on two dimensional TLC using chloroform-methanol-ammonia (25%) (65:25:4 and 35:60:5) as developing solvents. Phospholipids were identified on TLC plates by covering the silica gel plate (excepting the marker lane) with glass and spraying the exposed area with 1% iodine in chloroform, ninhydrin and molybdenum (14) reagents. Phosphorus in the corresponding unsprayed portions was estimated by scraping the gel from the plates and direct digestion with perchloric acid. The phospholipids were further identified by cochromatography on TLC plates with authentic markers. Large amounts of

TABLE IV

Phospholipid Composition of Subcellular fractions of Fourth Instar Larvae, *Culex pipiens fatigans*

Phospholipids	Per cent phospholipids		
	Cell debris and nuclei	Mitochondria	Microsomes
Cardiolipin	5	3	—
Phosphatidyl ethanolamine	52	56	61
Phosphatidyl choline	17	24	20
Sphingolipid	4	3	3
Lysophosphatidyl ethanolamine	10	4	4
Lysophosphatidyl choline	4	—	—
Phosphatidyl inositol	4	4	6
Phosphatidyl serine	3	6	5

phospholipids were isolated by preparative TLC with chloroform-methanol-ammonia as developing solvent. Identification was further confirmed when considered necessary by detailed analysis of the isolated lipids. Analytical methods for mild alkaline hydrolysis of the lipids and estimation of glycerol, fatty acid and phosphorus on the lipid samples were as described previously (8). Amino nitrogen and nitrogen were determined by the methods of Lea and Rhodes (15) and Lang (16) respectively. The presence of phosphonolipids was investigated by subjecting the total phospholipids, PE, PC and sphingolipids to strong hydrolysis with 5 N HCl for 42 hr at 120 C in a sealed tube under which conditions the C-P bond is stable (17).

Fatty acids of the principal phospholipid classes were isolated and methylated with 0.8 N anhydrous methanolic HCl according to the method of Gray (18). For positional distribution of fatty acids in the major phospholipids of the insects, the experimental procedure for incubation of the purified lipids with *Culex* and snake venom enzymes, isolation and methylation of fatty acids was as described elsewhere (19). The fatty acid methyl esters were subjected to gas liquid chromatography on a Packard gas chromatograph with argon ionization detector using a 6 ft column packed with EGSS-X on chromosorb W, 60/80 mesh at 190 C with a gas flow rate of 60 ml/min. Fatty acid methyl esters from Applied Science Laboratories, State College, Pa. were used as standards in addition to the relative retention times for identification of the peaks. Unsaturation in the fatty acid esters was further confirmed by bromination of the methyl esters and subsequent gas chromatography. Area within the peak was measured by triangulation.

Plasmalogens were determined following the method of Williams et al. (20). Acid and alkali stable lipids were isolated after the hydrolytic procedure of Preis et al. (21). Sphingosine was liberated from sphingolipids (SPL) by hydrolysis for 18 hr at 70 C in aqueous methanolic HCl after the method of Gaver and Sweeley (22) and estimated according to the method of Lauter and Trams (23).

RESULTS

The lipid composition of different developmental stages of the insects is shown in Table I. Egg rafts were found to contain greatest amounts of lipids. There was a decrease in both neutral lipids and phospholipids in fourth instar larvae compared with those of eggs. Lipid content increased in pupae and adults, mainly due to increase in neutral lipid fraction.

Qualitative examination of the nature of neutral lipids of the fourth instar larvae by TLC revealed that the major lipid of the insects was triglyceride. Diglyceride was present in significant amounts while monoglyceride, cholesterol, cholesterol ester and free fatty acids formed minor components of the insect lipids.

The phospholipid composition of different developmental stages is given in Table II. The major phospholipids of the insects were phosphatidyl ethanolamine (PE) and phosphatidyl choline (PC) at all stages as revealed by Dawson hydrolysis and other analytical methods detailed above. In egg rafts these two lipids were in about equal proportions while in other stages PE was present in highest amounts. No plasma-

TABLE V

Fatty Acid Composition of Phospholipids of Fourth Instar Larvae, *Culex pipiens fatigans*

Fatty acid	Total phospholipid	PE[a]	PE 1	PE 2	PC	PC 1	PC 2
14:0	1	1	1.5	---	1.5	4.4	1
14:1	4.0	3.0	---	8.1	8.0	2.2	8.8
16:0	18.4	26.5	48.0	5.4	17.0	28.5	6.0
16:1	39.4	34.4	31.5	41.0	44.0	48.0	45.8
18:0	1.0	1.0	1.7	---	---	1.0	---
18:1	31.6	32.0	16.5	37.1	23.0	15.0	31.6
18:2	4.7	2.8	1.2	8.0	6.0	1.0	8.0

[a]Abbreviations: see Table II.

logens or phosphonolipids could be detected in the major lipids. Among the minor lipids were phosphatidic acid, cardiolipin, lysophosphatidyl ethanolamine, phosphatidyl inositol, lysophosphatidyl choline, phosphatidyl serine and sphingolipid (SPL). The SPL moved along with the lower portion of the PC spot on TLC with chloroform-methanol-water (65:25:4) but separated from PC when chloroform-methanol-ammonia (65:25:4) was used as the developing solvent. The SPL was mild alkali and acid stable (21) and was ninhydrin positive. On sealed tube hydrolysis of the lipid with 5 N HCl at 120 C for 42 hr and subsequent chromatography of the aqueous portion, ethanolamine was found as the sole amine-containing compound. Phosphorus was released as inorganic phosphate under these conditions suggesting the absence of phosphonolipids (17). The lipid was subjected to hydrolysis with aqueous methanolic HCl (22) and extracted with chloroform. The extract, on TLC in chloroform-methanol-water (50:21:3), was found to contain sphingosine which cochromatographed with authentic sphingosine. This was later estimated (23) and the sphingosine-P molar ratio in the lipid was found to be 0.9. The phospholipid was therefore identified as ethanolamine-containing sphingolipid.

The subcellular distribution of the lipids in fourth instar larvae is presented in Table III. Highest amounts of lipids were found in cell debris and nuclei fraction and the supernatant had only minor amounts. The phospholipid content of mitochondrial fraction was much higher than that of microsomes while the neutral lipid was higher in microsomes.

The phospholipid composition of the subcellular fractions of larvae is given in Table IV. The major lipids of the fractions were again PE and PC. Lysolipids, in particular lysophosphatidyl ethanolamine (LPE), were present in higher amounts in cell debris and nuclei compared with other fractions. Cardiolipin was present only in cell debris and mitochondria and could not be detected in microsomes.

The fatty acid composition of the phospholipids and their positional distribution in PE and PC are shown in Table V. The major fatty acids of the total phospholipids of the insects were palmitic (16:0), palmitoleic (16:1) and oleic acids (18:1) of which 16:1 was present in highest amounts. Among the minor fatty acids were myristic (14:0), myristoleic (14:1), stearic (18:0) and linoleic acids (18:2). Similar distribution of fatty acids was seen in the major phospholipids PE and PC with the unsaturated fatty acids 16:1 and 18:1 generally in higher amounts at the 2 position, although considerable amounts of these were also present in 1 position. The saturated fatty acid palmitic acid (16:0) was predominantly located in the 1 position of the phospholipids. Among the minor fatty acids 14:1 and 18:2 were in higher amounts at the 2 position.

Table VI presents fatty acids of the major phospholipids of subcellular components. In all subcellular fractions the major fatty acids were again 16:0, 16:1 and 18:1. Cell debris PE resembled mitochondrial PE in fatty acid composition but microsomal PE differed to some extent from that of the other fractions as well as total PE (Table V) by having higher 16:0 and lower 16:1 and 18:1. The cell debris LPE had higher 18:1 and lower 16:0 and 16:1 compared with PE. This composition differed from that of PE 2 which suggested that cell debris LPE did not arise on hydrolysis of PE at 1 position by the insect phospholipase A (19). No major differences were seen in PC fatty acids of the different subcellular fractions. However, less 16:1 and more 18:1 were found in the PC of subcellular fractions compared with that of total PC.

DISCUSSION

The data presented in this investigation

TABLE VI

Fatty Acid Composition of Major Phospholipids of Subcellular Fractions of Fourth Instar Larvae, *Culex pipiens fatigans*

Fatty acid	Cell debris and nuclei			Mitochondria		Microsomes	
	PE[a]	LPE	PC	PE	PC	PE	PC
14:0	1.0	< 1	< 1	< 1	< 1	1.7	1.0
14:1	4.4	< 1	2.9	1.2	3.2	6.3	1.5
16:0	23.3	15.1	20.1	26.0	21.2	40.4	25.0
16:1	34.2	25.7	29.0	27.5	33.2	22.4	29.3
18:0	1.0	1.7	3.0	3.0	2.6	2.2	2.5
18:1	32.7	46.3	32.0	37.2	29.7	23.6	30.7
18:2	4.0	10.2	11.2	5.6	9.2	3.1	9.7

[a]Abbreviations: see Table II.

(Table I) suggest that in *C. pipiens fatigans* neutral lipids and phospholipids were utilized during development of the eggs. The higher amounts of neutral lipids in pupae and adults could be due to increased synthesis of these lipids. Lang (24) has observed loss of lipids during pupal stage in *C. pipiens fatigans* and *C. pipiens molestus*. Fast and Brown (25) reported a 20% loss of phospholipids when larvae were subjected to starvation by exposure to distilled water for 24 hr. In the present study the phospholipids do not seem to alter significantly either in pupae or adults when compared with larvae. Study of turnover of phospholipids with labeled precursors would throw more light on the dynamic aspects of metabolism of lipids in the insects.

Among the phospholipids, PE was present in highest amounts in *C. pipiens fatigans* which is generally considered characteristic in Diptera (26,27). PE and PC comprise over 75% of the total phospholipid of the species. Fast and Brown (25) reported the presence of sphingomyelin in *Aedes aegypti*. No sphingomyelin could be detected in *C. pipiens fatigans*. However, an ethanolamine-containing SPL has been identified among the lipids which appears to be similar in structure to one recently reported in several insects (17,27,28). Another significant observation is the presence of higher amounts of lysolipids particularly LPE in *Culex*. These lipids were not found in significant amounts when the insects were processed at room temperature, probably because of high activity of phospholipase B (6) which degrades the lysolipids. Therefore, care was exercised in processing the insect material especially the homogenates during preparation of subcellular fractions under cold conditions.

Nearly 50% of the lipids of larvae were found to be distributed in cell debris and nuclei fraction. This together with observation of higher amounts of lysolipids in this fraction warrants further study of the function of these lipids. Whether lysolipids have any role in lysis of membranes of the old cuticle during larval molting (29) deserves investigation.

Data on phospholipid composition of subcellular fractions of insects are scanty. The few studies suggest that the distribution pattern in Diptera (30,31) differs from that of other insect species (32,33). Distribution of different phospholipids among subcellular fractions in larvae of *Culex* differed from that reported for adult housefly. In housefly (30) PE was present in lower amounts and LPE in higher levels in microsomes compared with the other fractions. Crone (34) and Khan and Hodgson (30) reported that mitochondria of adult housefly *Musca domestica* has small amounts of cardiolipin. However, recently Chan (31) observed that cardiolipin comprises ca. 25% of total phospholipids of housefly mitochondria. In the present investigation mitochondria prepared from fourth instar larvae both by the procedure described under "Materials and Methods" and that of Chan (31) contained low amounts of cardiolipin.

As reported by Fast and Brown (25) in *Aedes*, 16:1 was the major fatty acid in the phospholipids of *Culex*. This was also the fatty acid present in highest amounts in the major phospholipids PE and PC. In general fatty acid distribution in the major lipids corresponded to that in animal species in that saturated fatty acids were predominantly attached to the 1 position and unsaturated ones were in higher amounts at the 2 position.

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Effect of ATP on the Microsomal Desaturation of Unsaturated Fatty Acids

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ABSTRACT

The effect of ATP on the microsomal desaturation of linoleic acid to γ-linolenic acid was studied in a system in vitro with the following results: (1) preincubation of rat liver microsomes with ATP alone in N_2 or in the presence of CoA and Mg^{++} followed by subsequent incubation with 1-^{14}C-linoleic acid plus NADH in O_2 resulted in enhancement of 1-^{14}C-linoleic acid desaturation when compared with control samples in which no preincubation was performed; (2) the preincubation of the microsomes with ATP, Mg^{++} and CoA in the presence of 1-^{14}C-linoleic acid decreased the desaturation of the labeled acid to γ-linolenic acid upon subsequent incubation with NADH, as a consequence of incorporation of the acid into the microsomal lipids; (3) the increase of linoleic acid desaturation depended on the ATP concentration during preincubation and followed a sigmoidal curve. It was specific for ATP, and neither GTP, CTP, ADP nor AMP produced a similar effect. However, GTP or CTP could replace ATP as a cofactor in the microsomal desaturation of free linoleic acid to γ-linolenic, suggesting that directly or indirectly they may activate conversion of the free acid to linoleyl-CoA; (4) preincubation of microsomes with ATP activated the acylation of CoA. However, this activation showed no quantitative correlation with enhancement of the desaturation reaction; (5) addition of ATP also stimulated conversion of linoleyl-CoA to γ-linolenic acid. This enhancement was not related to inhibition of the linoleyl-CoA hydrolase; (6) however, in spite of these results, preincubation with ATP did not increase the initial velocity of linoleic acid or linoleyl-CoA desaturation; (7) preincubation of microsomes with ATP also increased the 6-desaturation of oleic acid and α-linolenic acid but did not increase the 9-desaturation of palmitic and stearic acid.

INTRODUCTION

The oxidative desaturation of saturated and unsaturated fatty acids to synthesize new fatty acids has recently been extensively studied. It is produced by microsomal enzymes in the presence of NADH, NADPH and oxygen, prior conversion of the fatty acid into acyl-CoA. In other reports (1,2) we outlined a general scheme of some regulatory mechanisms that may be contributing to the control of this reaction. In outlining this scheme it was specially stressed that the desaturation activity was studied in a microsomal system in which the contribution of other collateral reactions to the desaturation reaction is very important. Such influencing reactions might be: prior activation of the substrate to the CoA thioester, the competitive desaturation reactions of other fatty acids, incorporation of the substrate and product into microsomal lipids, the microsomal electron transport (3) and all reactions that alter the levels of key metabolites and cofactors such as NADH or NADPH, CoA and ATP. However, it must be remarked that while some of these factors may be relevant to a study in vitro, they may be of negligible importance when considered in the context of the whole animal. In this paper we have investigated the effect of ATP on the desaturation of linoleic acid to γ-linolenic acid and the possible role played by other collateral reactions.

MATERIALS AND METHODS

Labeled acids were purchased from Radiochemical Centre, Amersham, England. 1-^{14}C-linoleic acid (52.9 mC/mmole) was 98% radiochemically pure and contained 2% *cis-trans* unsaturated acid. 1-^{14}C-oleic acid (36.9 mC/mmole) was 95% radiochemically pure and contained 5.3% elaidic acid. 1-^{14}C-α-linolenic acid (41.5 mC/mmole) was 98% radiochemically pure and contained 2% *cis-trans* isomer. 1-^{14}C-stearic acid (15.0 mC/mmole) was 99% radiochemically pure. 1-^{14}C palmitic acid (29.6 mC/mmole) was 98% radiochemically pure.

[1]The authors are members of the Carrera del Investigador Cientifico of the Consejo Nacional de Investigaciones Cientificas y Tecnicas.

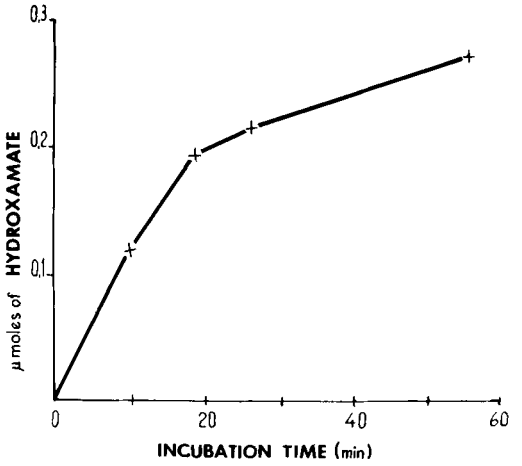

FIG. 1. Effect of incubation time on hydroxamate formation. Incubation of 3 mg of microsomal protein and 2 µmoles of ammonium linoleate at 37 C in N_2. ATP, CoA, $MgCl_2$, NaF, cysteine and hydroxylamine were included at concentrations indicated in the methods section.

ATP (disodium salt, 99% pure) and ADP (97% pure) were purchased from Sigma Chemicals Co., U.S.A.; whereas CTP, GTP, AMP, NADH and other cofactors were products of Fluka A.G., Buchs, Switzerland. 1-^{14}C-linoleyl-CoA was prepared by the procedure of Kornberg and Pricer (4) and the purity checked by paper chromatography (5).

Throughout the experiments fresh livers were used from male rats from the Institute strain weighing ca. 160 g and maintained on a complete diet. The livers were immediately homogenized in the cold with a solution of 0.15 M KCl, 1.5 mM glutathione, 62 mM phosphate buffer (pH 7) and 0.25 M sucrose.

The microsomal fraction (10,000-100,000 x g precipitate) of the livers was separated by the conventional differential centrifugation method that has been previously described (6).

Preincubation of Microsomes and Measurement of Linoleic Acid Desaturation

The effect of ATP and other nucleotides on the microsomal desaturation of labeled linoleic acid to γ-linolenic acid was investigated by preincubation of the microsomes with the factor to be tested. When not specified, the preincubation was performed at 35 C for 15 min in N_2 with 5 mg microsomal protein separated in the usual way in the absence of $MgCl_2$. The preincubation solution, when not specified, contained 2.5 µmoles of ATP, 4.5 µmoles of glutathione, 125 µmoles of NaF, 1 µmole of nicotinamide and 125 µmoles of phosphate buffer (pH 7.0) in a total volume of 3 ml of a 0.15 M KCl, 0.25 M sucrose solution.

TABLE I

Stimulation of Linoleate Desaturation by Prior Incubation of Microsomes With ATP[a]

Additions in preincubation	Desaturation %
None	3.8
ATP (4 µmoles)	15.0
$MgCl_2$ (15 µmoles)	4.0
CoA (0.2 µmoles)	3.1
ATP + $MgCl_2$	12.3
ATP + $MgCl_2$ + CoA	15.3
1-^{14}C-linoleic acid (5 nmoles)	5.5
1-^{14}C-linoleic acid + ATP + $MgCl_2$	3.5
1-^{14}C-linoleic acid + CoA	5.0
Without preincubation	9.5
Without preincubation[b]	0.0

[a]Microsomes (5 mg) were preincubated in phosphate buffer (pH 7.0) for 15 min at 35 C in nitrogen with the corresponding cofactors as described. After preincubation the solution was chilled. NADH (2.5 µmoles); 1-^{14}C-linoleic acid (5 nmoles) and the complementary cofactors ATP, $MgCl_2$ and CoA were added when necessary. The solution was incubated 20 min at 35 C in oxygen. Results are expressed as per cent conversion of linoleic acid to γ-linolenic acid.

[b]Incubation in the absence of ATP and $MgCl_2$.

To measure the desaturation of linoleic acid to γ-linolenic acid or of the other acids tested, the preincubation solution was chilled and a supplementary amount of ATP, in order to produce a total of 2.5 µmoles, was immediately added when necessary along with 15 µmoles of $MgCl_2$, 0.2 µmoles of CoA, 2.5 µmoles of NADH and 5 nmoles of 1-^{14}C-linoleic acid or of the other labeled acids tested. The solution was gassed with O_2 and incubated for 20 min at 35 C. The incubation was stopped by the addition of 2 ml of 10% methanolic KOH. After a 40 min saponification at 85 C the solution was acidified and the fatty acids were extracted and esterified with 3 N methanolic HCl for 3 hr at 68 C. The per cent conversion of linoleic acid to γ-linolenic acid was measured by gas-liquid radiochromatography in a Pye apparatus with a proportional counter as described previously (6).

A similar control incubation was performed with the same cofactors at 35 C in O_2 for 20 min, however without preincubation, using the same procedure for the other acids tested. When not specified, the desaturation of 1-^{14}-linoleyl-CoA was measured by incubation under conditions similar to those used with the free acid, but in the absence of ATP, $MgCl_2$ and CoA. All data collected are the means of at least two experiments.

Study of Linoleyl-CoA Synthesis by Hydroxamate Formation

The microsomes used for the investigation of

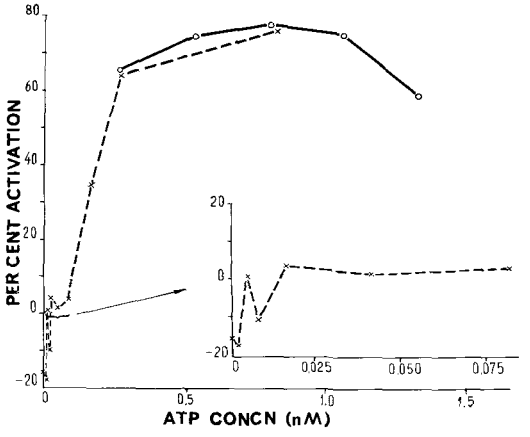

FIG. 2. Effect of ATP concentration in the preincubation mixture of linoleic acid desaturation to γ-linolenic acid. 5 mg of microsomal protein were preincubated under conditions described in the methods section at 35 C for 15 min in N_2 with ATP included at the concentration indicated. The subsequent incubation was at 35 C for 20 min in O_2 after the addition of 15 μmoles $MgCl_2$ 0.2 μmoles CoA, 2.5 μmoles NADH and 5 nmoles 1-^{14}C-linoleic acid. A supplement of ATP was added to reach a final concentration of either 1.33mM (•—•) or 0.83 mM (x - - x). The results are the mean of duplicate samples and are expressed as per cent activation compared with the nonpreincubated control sample.

linoleyl-CoA synthesis were separated in the usual way except for substitution of 1 M Tris-HCl buffer (pH 7.4) for the usual phosphate buffer. Linoleyl-CoA synthesis, when not specified, was followed by trapping the linoleyl-CoA with hydroxylamine (7).

In the experiments involving preincubation, the reaction mixture contained 50 μmoles of Tris-HCl buffer (pH 7.4), 5 μmoles of NaF, 22.5 μmoles of cysteine, 1 to 5 mg of microsomal protein and the tested cofactors in a total volume of 0.7 ml. After preincubation for 15 min in N_2 at 37 C, 200 μmoles of hydroxylamine and supplementary amounts of ATP, $MgCl_2$ and CoA were added in order to produce a total of 10 μmoles, 4 μmoles and 0.5 mg respectively. The reaction was initiated by the injection of 2 μmoles ammonium linoleate. The mixture was incubated in N_2 in a total volume of 1 ml from 10 to 60 min as specified. In the controls all components of the mixture were incubated for the specified time periods without preincubation. The incubation was stopped with 0.1 ml of 60% (W/V) $HClO_4$ and 1 ml of water. After centrifugation the precipitate was extracted with 2.5 ml of 96% ethanol. After addition of 0.3 ml of 10% (W/V) $FeCl_3$ in 0.1 N HCl the extinction at 540 nm was read using a Zeiss spectrophotometer. An extinction of 0.425 corresponded to 1 μmole of hydroxamate. The effect of time of incubation on the number of μmoles of hydroxamate formed is shown in Fig. 1. Within the range of 1-5 mg of microsomal protein, the acylation of linoleic acid was proportional to the quantity of microsomes present.

TABLE II

Effect of Gas Phase and Time of Preincubation With ATP on the Desaturation of Linoleic Acid[a]

Preincubation conditions		Conversion
Time (min)	Gas phase	%
0	N_2	25.5
2	N_2	29.1
4	N_2	35.8
6	N_2	37.0
10	N_2	36.9
15	N_2	37.2
20	N_2	37.2
15	Air	28.0

[a]5 mg microsomal protein were preincubated with 2.5 μmoles ATP, 15 μmoles $MgCl_2$ and other cofactors described in the methods section without CoA and NADH at pH 7.0 and 35 C. The incubation was immediately performed after addition of NADH, CoA and 5 nMoles of 1-^{14}C-linoleic acid in a total volume of 3 ml for 20 min at 35 C in air.

Study of Linoleyl-CoA Synthesis by Separation of 1-^{14}C-Linoleyl-CoA.

Four milligrams microsomal protein were incubated 20 min at 30 C with 4 μmoles of ATP or the other specified nucleotides, with the following additions in μmoles: cysteine, 12; CoA, 0.4; NaF, 20; $MgCl_2$, 6; phosphate buffer (pH 7.5), 40; and ammonium-1-^{14}C-linoleate, 0.8 (3.92 x 10^5 cpm). The total volume was 1.2 ml. The reaction was stopped with 0.6 ml of 0.3 N $HClO_4$, and the linoleyl-CoA was separated by the method of Creasey (8). The mixture was centrifuged and the precipitate extracted three times with 0.1 N $HClO_4$, three times with 80% ag. thanol and three times with ethanol-ether (1:1 V/V). The linoleyl-CoA was extracted three times with 1 ml ethanol-water-isopropanol (1:1:1 V/V/V) and counted in a scintillation counter.

Linoleyl-CoA Hydrolase

The microsomal hydrolysis of linoleyl-CoA was investigated by incubating 5 mg microsomal protein and 5 nmoles 1-^{14}C-linoleyl-CoA at 35 C for 5 min in air. The incubation solution contained the same cofactors used for the measurements of desaturation except for $MgCl_2$, CoA and NADH. Increasing concentrations of ATP were tested. The free acid

TABLE III

Comparative Effect of Different Nucleotides and L-αGlycerophosphate in the Desaturation of Linoleic Acid to γ-Linolenic Acid[a]

Additions in Preincubation (μmoles)		Additions in Incubation (μmoles)		Conversion %
None		ATP	2.5	32.4
ATP	0.005	ATP	2.495	23.4
	2.5		---	50.9
ADP	0.005	ATP	2.5	25.6
	2.5		2.5	36.9
AMP	2.5	ATP	2.5	29.6
GTP	1.0	ATP	2.5	24.4
	2.5		2.5	27.9
	4.0		2.5	27.8
CTP	2.5	ATP	2.5	27.2
L-αglycerophosphate	1.0	ATP	2.5	18.3
	2.5		2.5	12.4
Without preincubation		ATP	2.5	33.3
		ADP	2.5	32.8
		AMP	2.5	9.9
		GTP	1.0	32.5
			2.5	36.9
		CTP	2.5	34.8

[a]5 mg microsomal protein were preincubated for 15 min in N_2 at 35 C with the specified cofactors. The subsequent incubation was performed after addition of 15 μmoles $MgCl_2$; 0.2 μmoles CoA; 2.5 μmoles NADH; 5 nmoles 1-^{14}C-linoleic acid and complementary amounts of ATP to reach a total amount of 2.5 μmoles. The total volume was 3 ml.

liberation was corrected by comparison with similar samples in which 1 mg albumin was substituted for the microsomes. The incubation was stopped by the addition of 0.1 ml of 0.5 N H_2SO_4. The free labeled acid was extracted three times with 2 ml of hexane, evaporated and counted in a Packard scintillation counter. The per cent hydrolysis was calculated.

RESULTS AND DISCUSSION

Effect of ATP on Linoleic Acid Desaturation

The oxidative desaturation of linoleic acid to γ-linolenic acid occurs in the microsomes and requires oxygen, NADH or NADPH, and prior activation of the free acid to linoleyl-CoA. This synthesis is produced by an acyl-CoA synthetase that requires ATP and subsequently converts it to AMP and pyrophosphate (7). Under our standard experimental conditions, adjusted to measure the desaturation of free linoleic acid to γ-linolenic acid, the conversion of linoleic acid to linoleyl-CoA is produced in the same microsomal system by the addition of ATP and $MgCl_2$. The per cent desaturation of free linoleic acid in this medium is quantitatively similar to that of linoleyl-CoA.

However when liver microsomes were preincubated with ATP, CoA and Mg for 15 min in N_2 at 35 C in the absence of NADH or NADPH and then the reduced coenzyme and 1-^{14}C-linoleic acid were added and incubated in O_2 during 20 min, desaturation of linoleic acid was greatly stimulated when compared with the nonpreincubated control (Table I). This stimulation was also found when only ATP was added and CoA and Mg were not present during the preincubation. Furthermore the preincubation of microsomes without ATP decreased the desaturation compared with the nonpreincubated sample, thus showing that ATP not only stimulated but also protected the reaction. Table I also shows that addition of the labeled linoleic acid during preincubation of the microsomes in the presence or absence of ATP reduced its conversion to γ-linolenic acid. This effect is apparently due to incorporation of the substrate into microsomal lipids, decreasing the amount of 1-^{14}C-linoleic acid available for a subsequent desaturation.

The stimulation of linoleic acid desaturation to γ-linolenic acid by prior incubation of the microsomes with ATP was reasonable rapid

TABLE IV

Comparative Effect of GTP and ATP on Microsomal Linoleyl-CoA Synthesis[a]

Additions in incubation	Hydroxamate
ATP	0.0166 ± 0.0010
GTP	0.0038 ± 0.0002

[a]2.5 mg of microsomal protein were incubated 20 min at 37 C in 1 M Tris-HCl buffer (pH 7.4) with the cofactors and under conditions described in the methods section. 10 μmoles of ATP or GTP were added when specified. The results are the mean ± SE of samples in quadruplicate and are expressed in μmoles of hydroxamate formed per min per mg of protein.

(Table II). Substitution of air for N_2 decreased the enhancing effect of ATP (Table II) probably because of the oxidation of some oxygen-sensitive component of the desaturation reaction.

Since ATP stimulated the desaturation of linoleic acid it was of interest to investigate the effect of variations in ATP concentration. As may be seen in Fig. 2 the prior incubation of microsomes with increasing concentrations of ATP demonstrated an apparent dependence on the nucleotide concentration. In all cases the final concentration of ATP during the incubation was maintained constant (1.33 mM) whereas the concentration of ATP in the preincubation mixture was varied from 0.26 to 1.33 mM. Under these conditions and in the presence of 5 mg of microsomal protein the highest level of desaturation was attained at 0.8 mM ATP.

In a second experiment the effect of vary low concentrations of ATP on the preincubation of 5 mg of microsomal protein was studied. Here the final total ATP concentration of the incubation mixture was maintained at 0.83 mM (2.5 μmoles in 3 ml) (Fig. 2). Under these experimental conditions, a definite relationship between ATP concentration and the stimulation of fatty acid desaturation was again

TABLE V

Effect of ATP, CTP, and GTP Upon the Acylation of the CoA in Liver Microsomes[a]

Nucleotide added	Radioactivity in linoleyl-CoA (cpm)
None	273
ATP	10,160
CTP	3,670
GTP	1,530

[a]4 mg microsomal protein incubated 20 min at 30 C in phosphate buffer (pH 7.4) as described in the methods section.

demonstrated. Very low concentrations of ATP resulted in erratic data, with lower desaturation than those obtained without preincubating the microsomes. Concentrations of about 0.02 mM apparently protected the microsomal enzymes from inactivation during preincubation, whereas higher amounts of ATP were necessary to elevate the desaturation above normal levels.

A remarkable stimulation of linoleic acid desaturation to γ-linolenic acid was also found when concentration of ATP was increased from 0.8 mM to 100 mM during normal incubation of the microsomes in desaturating conditions. The per cent conversion was increased from 7.0 to 21.0.

Effect of Other Nucleotides and L-αGlycerophosphate on Linoleic Acid Desaturation

The remarkable stimulation of linoleic acid desaturation evoked by preincubation of the microsomes with ATP may be produced by different mechanisms. Therefore, both to get more information of the problem and to test the specifity of the ATP effect, a comparative experiment with ATP, ADP, AMP, GTP, CTP and L-αglycerophosphate was performed. The results are summarized in Table III and illustrate that neither ADP, AMP, GTP, CTP nor L-αglycerophosphate is able to increase the desaturation ability of the microsomes.

TABLE VI

Effect of ADP, CTP and GTP on the Linoleyl-CoA Synthesis in Liver Microsomes[a]

Nucleotides added	μmoles	Radioactivity in linoleyl-CoA cpm
None	4	105
ATP	4	1,384
CTP	4	295
GTP	4	192
ADP	4	852
ADP + CTP	4 + 4	413
ADP + GTP	4 + 4	498

[a]Experimental conditions similar to Table V but in buffer Tris-HCl (pH 7.4).

TABLE VII

Stimulation of Linoleyl-CoA Synthesis by Preincubation With ATP[a]

Additions in preincubation	Hydroxamate
ATP	0.0040 ± 0.0001 (P<0.01)
ATP + CoA + $MgCl_2$	0.0042 ± 0.0002 (P<0.01)
With preincubation	0.0034 ± 0.0001

[a]3 mg of microsomal protein were preincubated for 15 min at 37 C in N_2 with 5 μmoles of NaF, 22.5 μmoles of cysteine, 50 μmoles of Tris-HCl buffer (pH 7.4) and 2 μmoles of ATP or 2 μmoles of ATP + 0.5 mg of CoA + 4 μmoles of $MgCl_2$, where specified. The samples were subsequently incubated for 20 min at 37 C in N_2 with 200 μmoles hydroxylamine, 2 μmoles ammonium linoleate, and supplementary amounts of ATP, CoA and $MgCl_2$ to reach 10 μmoles, 0.5 mg and 4 μmoles respectively in all the tubes. The control without preincubation contained the same amount of all components. Results are expressed as μmoles of hydroxamate formed per min per mg of protein ± SE of the mean.

Hence it can be concluded that the ATP-evoked stimulation of desaturation is specific and can not be merely ascribed to an unspecific effect of nucleotides. However, GTP and CTP were able to replace ATP and maintain the desaturation of linoleic acid at the normal level. This result would suggest that CTP and GTP are able to provide, directly or indirectly, the energy for conversion of linoleic acid to linoleyl-CoA thus facilitating its subsequent desaturation. However, Pande and Mead (9) were unable to show the existence of a GTP-dependent enzyme able to synthesize oleyl-CoA in their preparations of rat liver microsomes. Therefore in order to better understand the effect of ATP on the fatty acid desaturation it was important to investigate first the effect of different nucleotides on the microsomal synthesis of linoleyl-CoA.

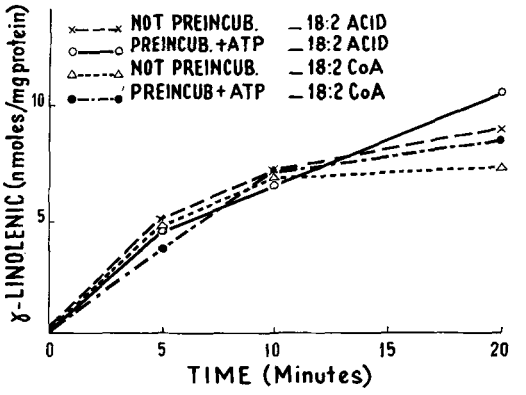

FIG. 3. Time course of desaturation of linoleic acid by rat liver microsomes: ○—○ preincubated with ATP for 15 min at 35 C in N_2 or x - - x nonpreincubated, and linoleyl-CoA by ●-·-·-● preincubated or △---△ nonpreincubated microsomes. 1 mg microsomal protein and 100 nmoles labeled substrate incubated in a total volume of 1 ml at 35 C in O_2.

Effect of ATP, CTP, and GTP on the Acylation of CoA

The synthesis of linoleyl-CoA by rat liver microsomes was studied by measuring the hydroxamate formed by the method of Bar-Tana and Shapiro (10) and also by extracting and counting the 1-^{14}C-linoleyl-CoA formed by the method of Creasey (8).

In Table IV it is shown that hepatic microsomes are able to employ GTP directly or indirectly for linoleyl-CoA synthesis. However the hydroxamate formed in this experiment was only one-fourth of that synthesized in the presence of ATP. Besides, the incubation of the liver microsomes with 1-^{14}C-linoleic acid in phosphate buffer followed by subsequent separation of the acyl-CoA precipitate and counting by the method of Creasey (8) also showed a measurable incorporation of the radioactivity in

TABLE VIII

Effect of ATP on Linoleyl-CoA Desaturation[a]

Additions in			
Preincubation	Incubation	Conversion	Activation[b]
ATP (2.5 μmoles)	18:2 CoA	27.4	25.1
ATP (2.5 μmoles)	18:2 acid + Mg^{++} + CoA	37.6	58.6
Without preinducation	18:2 CoA + ATP + Mg^{++} + CoA	21.9	---
	18:2 acid + ATP + MG^{++} + CoA	23.7	---

[a]5 mg microsomal protein were preincubated as described in the methods section with ATP, when specified, for 15 min in N_2 at 35 C. The subsequent incubation was performed in O_2 for 20 min after the addition of 10 nmoles 1-^{14}C-linoleyl-CoA or 1-^{14}C-linoleic acid with 2.5 μmoles NADH and 2.5 μmoles ATP; 15 μmoles $MgCl_2$; and 0.2 μmoles CoA, when specified. The results are expressed as per cent conversion of linoleic to γ-linolenic acid in duplicate samples.

the precipitate either with ATP, GTP or CTP (Table V). However, again the efficiency of the conversion was much lower with GTP and CTP than with ATP. Considering that a GTP-specific acylating synthetase that cleaves the nucleotide to nucleoside diphosphate and P_i has been described in mitochondria by Rossi and Gibson (11), the low synthesis of linoleyl-CoA found with GTP compared with ATP could be attributed to a selective inhibition produced by the phosphate of the buffer. For this reason a similar experiment was performed in Tris-ClH buffer and this possibility was discarded because no further improvement in acylation was found. Therefore it was very improbably that GTP and CTP would activate directly the linoleyl-CoA synthesis.

The fact that replacement of ADP for ATP in the incubation of microsomes did not decrease the conversion of linoleic acid to γ-linolenic acid (Table III) led us to consider a possible acylation of CoA mediated through a conversion of ADP to ATP. GTP and CTP would contribute to this conversion. As may be seen in Table VI neither CTP nor GTP addition to ADP increased the synthesis of linoleyl-CoA. Therefore the mechanism of GTP and CTP contribution to free linoleic acid desaturation to γ-linolenic acid in liver microsomes is uncertain, as is the effect of ADP, and further investigation is needed. However, the study of possible products of nucleoside triphosphate transformation was hindered by existence of a very active nucleoside triphosphatase which splits ATP, CTP and GTP in the microsomes (12).

All these results show that the effect of ATP is not only rather specific in the microsomal synthesis of linoleyl-CoA, but also in the stimulation of the microsomes for linoleic acid desaturation to γ-linolenic acid. Therefore it was considered probable that the effect of ATP on the increase of linoleic acid desaturation could be related in some way to acylation of the CoA. One possibility was that preincubation of the microsomes by ATP led to faster formation of linoleyl-CoA. This mechanism could be consistent with the scheme proposed by Bar-Tana and Shapiro (10) for the synthesis of palmityl-CoA, in which ATP activates the formation of an Enzyme-CoA complex that further consumes a mole of ATP to produce a final acyl-CoA, AMP and pyrophosphate. To investigate this effect the microsomes were preincubated with ATP as described in the methods section and the increase of linoleyl-CoA synthesis was measured by the hydroxamate formation. Table VII shows an activation of hydroxamate formation when either ATP alone or together with $MgCl_2$ and CoA were preincubated with the microsomes. However, the activation of linoleyl-CoA synthesis produced by ATP under these conditions (15%) was lower than the stimulation of linoleic acid desaturation evoked by ATP (>37%). Therefore it is difficult to attribute all the stimulating effect of ATP on linoleic acid desaturation to an increase of linoleyl-CoA synthesis.

TABLE IX

Effect of ATP on the Linoleyl-CoA Hydrolase[a]

Additions, ATP, μmoles	Per cent hydrolyzed
---	13.1
0.5	16.3
1.0	13.3
2.5	15.5

[a]Five mg microsomal protein were incubated for 5 min at 37 C in air with 5 nmoles 1-^{14}C-linoleyl-CoA under the conditions described in the methods section. ATP was added when specified. The total volume was 3 ml. At the end of incubation the free fatty acids were extracted and counted. Results are the mean of duplicate samples.

TABLE X

Comparative Effect of ATP on Stearic, Palmitic, Oleic, Linoleic and α-Linolenic Acid Desaturation[a]

	Conversion		
	Preincubation		
Acid tested	With ATP	Without ATP	Without preincubation
Linoleic	26.5	17.7	20.0
Oleic	4.0	---	2.8
α-linolenic	56.4	35.9	44.4
Stearic	5.8	3.4	7.3
Palmitic	7.1	4.5	9.7

[a]Microsomes were preincubated for 15 min in N_2 at 35 C with or without ATP and immediately incubated as described in the methods section.

Comparative Effect of ATP on Linoleic Acid and Linoleyl-CoA Desaturation

To elucidate whether the effect of ATP was dependent on linoleyl-CoA synthesis, the microsomes were preincubated with ATP in the usual way and the desaturation of 1-^{14}C-linleyl-CoA was measured. The results are shown in Table VIII. A comparison of the experimental values with those of the controls, in which preincubation was not performed, demonstrated that preincubation with ATP not only enhanced the desaturation of the free acid but also that of the CoA derivative. These results may suggest that ATP can specifically increase the desaturation reaction in addition to producing its effect on linoleyl-CoA synthesis. However this effect could also be produced by inhibition of the microsomal linoleyl-CoA hydrolase by preincubation with ATP, thus bringing about a similar increase in level of desaturation as a result of an increased amount of available substrate. However, this hypothesis may be discarded after testing the effect of ATP on microsomal linoleyl-CoA hydrolysis. In this respect Table IX demonstrates that under the experimental conditions tested, ATP does not inhibit linoleyl-CoA hydrolase activity.

In all these experiments the conversion of linoleic acid to γ-linolenic acid was measured with low substrate concentration and in conditions near the equilibrium. Therefore it was important to investigate if ATP increased the amount of linoleic acid converted to γ-linolenic acid per mg of microsomal protein during increasing periods of incubation with high concentrations (0.1 mM) of substrate and low amounts of microsomes (1 mg). Microsomes preincubated and nonpreincubated with ATP were used. The results collected in Fig. 3 show that preincubation of the microsomes with ATP does not increase significantly the speed of desaturation of either the free acid or the linoleyl-CoA in saturating conditions of the enzyme. Therefore an increase in desaturation of linoleic acid was only found when the desaturating ability of the microsomes preincubated with ATP was measured at low substrate concentrations. We know that at low substrate levels other reactions, or even the amount of endogenous fatty acids, may modify the desaturation. Therefore our opinion is that ATP very probably provoked a modification of the microsomes. In this respect a chemical modification of the microsomal constituents is very probable and Marinetti (13) has shown that incubation of liver cells with different concentrations of ATP may modify the relative synthesis of phospholipids and triglycerides.

Effect of ATP on Stearic, Palmitic, Oleic and α-Linolenic Acid Desaturation

The effect of ATP was also tested on the desaturation of the following fatty acids: 1-^{14}C- stearic to oleic; 1-^{14}C- palmitic to palmitoleic; 1-^{14}C-α-linolenic to octadeca-6,9,12,15-tetraenoic (Table X). It was found that the preincubation of the microsomes with ATP increased the conversion of all the acids in which the new double bond is inserted between the 6 and 7 carbons, but decreased the conversion of the saturated acids to monoenoic acids with the double bond in 9-10 position. This result is quite interesting when it is considered that two different desaturating enzymes are involved in 9-desaturation and 6-desaturation (2). Besides, it has also been suggested that linoleic, α-linolenic and oleic acids are very probably desaturated by the same olefinase (2). Our results provide further evidence for this hypothesis.

Extent of the physiological significance of the stimulating effect of ATP is difficult to ascertain. The sigmoidal response to ATP concentration would insure extreme sensitivity of the desaturation reaction to a selected range of ATP concentration. However, as far as we know, actual concentration of ATP in the cell is between 2-8 mM and no convincing proof exists that the ATP synthesized in the mitochondria is not readily available to the extra-mitochondrial space of the cell. Moreover, mitochondria are unable to store ATP (14), and from the data of Heldt (15) it may be calculated that about 80% of the total adenine nucleotides of liver would be in the cytoplasm. Therefore since the active concentration of ATP is apparently lower than the concentration normally found in the cell, it is very probable that the same situation holds for the endoplasmic reticulum and the reaction would be fully active at physiological concentrations. In any event this conclusion needs experimental confirmation by simultaneous measurement of fatty acid desaturating activity and ATP concentration in the extra-mitochondrial space of the cell.

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Lipid Composition and Endogenous Respiration of Pig Heart Mitochondria

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ABSTRACT

Lipid composition and endogenous respiration of pig heart mitochondria were studied in parallel, since the level of endogenous respiration affects the oxidation of added substrates and therefore the regulation of oxidative phosphorylation; mitochondrial lipids can interfere either as substrates or as partner in the energy conservation mechanism. O_2 uptake kinetics were measured in presence of different additives: ATP, ADP, NAD^+ and hexokinase + glucose. The lipid composition of pig heart mitochondria was determined by chromatographic and spectrophotometric methods. Total lipids were 90% phospholipids; the main phosphatides were cardiolipin, phosphatidyl choline and phosphatidyl ethanolamine; the two latter were rich in plasmalogens. The main nonpolar lipids were triglycerides and free fatty acids. The fatty acid composition of total lipids, phospholipids, free fatty acids and triglycerides was determined by gas liquid chromatography. Mitochondrial lipids were characterized by a high content of unsaturation.

INTRODUCTION

Endogenous substrates in isolated mitochondria can affect the oxidation of added substrates (1) as well as their coupled oxidative phosphorylations (2-6) and respiratory control ratios (RCR) (7).

Pig heart mitochondria have a noticeable endogenous respiration. Pyruvate, glutamate, glutamine and alanine, the only substrates present in significant amounts, are mainly eliminated by washing (8). This treatment favors the participation of lipids in endogenous respiration as suggested by several authors (1,6,9-12). Besides, the mechanism of oxidative phosphorylation and its regulation are linked to the structure of the mitochondrial inner membrane, characterized by a specific lipid composition. We started therefore to study the lipid composition of pig heart mitochondria and its variations associated with endogenous respiration, with the further purpose to support our present studies on the mechanism of oxidative phosphorylation.

The lipid composition of pig heart mitochondria had been only partially studied previously. Phospholipid concentrations have been determined by Marinetti et al. (13) but Redfearn (14) gave simply a brief pattern of nonpolar lipids in nonphosphorylating mitochondria. Thus we were led to make a complete determination of all lipids in the phosphorylating pig heart mitochondria where endogenous respiration was studied. Differences were observed and are compared with previously reported results (13,14).

[1]Part of this work is included in "Thèse de Doctorat de Spécialité en Biochimie" de J. Comte, Lyon, June 26, 1970.

TABLE I

Endogenous Substrate Oxidation in Isolated Pig Heart Mitochondria

Additions	QO_2[a], natoms/mg proteins/mn
None	7.8
ADP	12.5
ATP	14.7
ATP + ADP	32

[a]Oxypolarographic measurements of O_2 consumption last 3 mn. Values are means of three determinations. 0.7×10^{-3} M ATP; 2×10^{-4} M ADP; 5 mg mitochondrial proteins.

METHODS

Preparation and Controls of Mitochondria

Pig heart mitochondria were prepared essentially according to Crane et al. (15). Proteins were determined by the quick biuret method (16). RCR was estimated according to Chance and Williams (17). Oxidative phosphorylation was measured in the presence of hexokinase + glucose; the oxygen consumption was measured manometrically and esterified phosphate by the Fiske and Subbarow method (18).

The standard reaction medium contained 0.016 M Tris-HCl, 0.112 M KCl, 0.006 M $MgCl_2$, 0.005 M phosphate. Other additions are given in the legends of the figures.

Extraction of Lipids

Lipids were extracted from the mitochon-

drial suspension in 0.25 M sucrose with chloroform-methanol 2:1 under nitrogen. For 800-1,000 mg mitochondrial proteins, 600 ml of solvent were used for the first extraction and 300 ml for each of two subsequent extractions. Each extraction was carried out for 24 hr. After filtration of mitochondrial fragments on a Millipore glass-fiber filter, solvents were evaporated and the lipid extract so obtained was freed of nonlipid impurities by the procedure of Folch et al. (19). The lipid part was dried in vacuum over KOH and P_2O_5 and determined by weighing.

Chromatographic Separations

The total lipids, 300 mg, in ethyl ether solution were applied to a column of Mallinckrodt silicic acid, 15 g, activated overnight at 110 C before use, and Celite, 7.5 g. Nonpolar lipids were eluted with 300 ml of hexane-ethyl ether (80:20) and phospholipids with 200 ml of chloroform-methanol (1:1) followed by 100 ml of methanol.

The nonpolar lipids were analyzed by thin layer chromatography (TLC) on Silica Gel G plates. For qualitative studies four solvent systems were used: I, hexane-ethyl ether-acetic acid (70:30:1) (20); II, hexane-ethyl ether-acetic acid (76:24:1); III, hexane-ethyl ether (80:20); IV, hexane-ethyl ether (50:50). The spots were visualized by spraying with rhodamine B and 2',7' dichlorofluorescein and viewing under ultraviolet light (21). Cholesterol and cholesteryl esters were revealed on the chromatogram by a ferric chloride spray reagent (22). For a further characterization small amounts of each compound were isolated by preparative TLC. Spots were detected with only rhodamine B and lipids were eluted from the adsorbent with ethyl ether. The quantitative TLC of nonpolar lipids was accomplished by charring and transmission densitometry according to Blank et al. (23) and Downing (24) with some modifications. The chromatogram, developed in solvent II, was sprayed with a solution of 20 g of ammonium sulphate in 4% H_2SO_4 and heated for 25 min at 225 C (25). It was scanned at 620 nm with a Chromoscan Joyce Loebl fitted with a thin layer chromatography search unit and a peak integrator. The proportion of each constituent was calculated from the corrected integrator units (24).

The phospholipids were separated on 20 x 20 cm Silica Gel H plates by two-dimensional TLC as described by Rouser et al. (26) with some modifications. After development of the chromatogram in solvent V, chloroform-methanol-water (65:25:4), a 5 mM $HgCl_2$ solution in 0.1 M acetic acid was sprayed on the

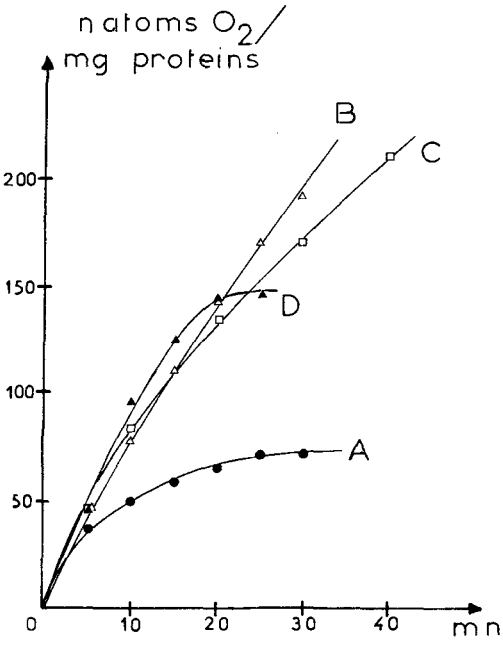

FIG. 1. Endogenous respiration kinetics. A: control (5 essays); B: with ATP 0.7 x 10^{-3}_{M} (5 essays); C: with NAD^+ 2 x 10^{-3} M (1 essay); D: with ATP + hexokinase + glucose (4 essays). The first reading (zero time) was effected after 10 min preincubation of mitochondrial proteins (10 mg); ATP or NAD were preincubated with mitochondria; hexokinase (100-150 Berger units) (34) were added at zero time in the Warburg cell, containing ATP.

lipid track according to Broekhuyse (27). After drying the plate in vacuum the $HgCl_2$-treated lipids were separated in the second direction with solvent VI, butanol-acetic acid-water (60:20:20). Each standard was chromatographed alone or in mixture with mitochondrial lipids. Individual phospholipids were detected by exposure to iodine vapors. Ammonium molybdate/perchloric acid (28), ninhydrin (28) and Wagner et al. (21) spray reagents were used for specific detection of substances containing phosphorus, primary and tertiary amino groups respectively; α-glycol groups were shown up by the periodate-Schiff reagent (29). For analysis phospholipids were isolated by preparative TLC and locating with iodine vapors. Scraped spots were applied to a silica gel column and eluted by chloroform-methanol (1:1) followed by methanol. Quantitative analysis of phospholipids was effected by phosphorus determination after two-dimensional TLC. Spots detected with iodine vapors were directly aspirated in a matrass, according to the procedure of Rouser et al. (26) and digested by adding 1 ml of a mixture of H_2SO_4, perchloric acid and 2.5% ammonium molybdate (3:6:1). Phosphorus was

TABLE II

Oxidative Phosphorylation of Endogenous Substrates[a]

Determinations	Measured values
Δ esterified P (μmoles)	0.72 ± 0.06
- Δ O (μ atoms)[b]	0.74 ± 0.10
P/O ratio[b]	0.97

[a]Values are averages of seven determinations followed by ±standard deviation; conditions are described in Methods: 10 mg mitochondrial proteins; 0.7×10^{-3} M ATP; hexokinase 100-150 Berger units (34).

[b]Manometric technique.

then determined by the Chain and Berenblum method (30) which permits elimination of silica gel in the lower aqueous solution without prior elution. Optical densities were measured with a Coleman Junior spectrophotometer at 700 nm. The values were corrected for gel blanks treated similarly. Usually spots from two chromatograms were pooled for minor components.

Analytical Methods

Acid hydrolysis was performed either on the total phospholipid fraction or on each purified phospholipid with 3 N HCl in sealed tube at 110 C for 16 hr. Fatty acids were extracted by ethyl ether and studied by gas liquid chromatography (GLC). The aqueous phase was examined by descending paper chromatography for the presence of ethanolamine, serine, inositol, glycerol and α-glycerol phosphate, in the solvent systems: butanol-acetic acid-water (65:10:25) and pyridine-ethyl acetate-water (2:5:5). The α-glycol groups were detected by periodate-benzidine reagent (31), amino groups by ninhydrin (28).

Triglycerides in benzene solution were saponified by addition of 5% methanolic KOH. After acidification fatty acids were extracted by ethyl ether and aqueous phase was examined by descending paper chromatography for glycerol presence.

The Liebermann-Burchard reaction (32) was performed on the cholesterol fraction, which was purified by TLC.

Analysis for coenzyme Q identification was performed by spectrophotometry. First the component was separated from nonpolar lipids by preparative TLC in solvent II. The spots were scraped and applied to a silica gel column, then eluted by heptane. After heptane evaporation the compound was dissolved in ethanol and spectrum was recorded from 200 to 360 nm.

For GLC study, fatty acids methyl esters were prepared with diazomethane. The analyses were carried out on a polar column of 25% diethylene glycol succinate at 170 C and on a nonpolar column of 20% Apiezon L at 210 C using a Chromagas CG_2 with flame ionization detector. Unsaturated fatty acids were hydrogenized by catalysis on platinum oxide (33). Fatty acid methyl esters were identified by comparison with standards, by a plot of log retention time against carbon numbers, by comparison with published values. Fatty acid amounts were estimated by peak area measurements.

Preliminary controls indicated that the peak areas were proportional to the amounts of fatty acid methyl esters.

Statistics

All standard errors of the means have been calculated.

RESULTS

Endogenous Respiration of Pig Heart Mitochondria

Only mitochondria capable of oxidative phosphorylation with theoretical P/O ratios were used (respiratory control ratios were about

TABLE III

Lipid Concentration of Pig Heart Mitochondria

Rf value[a]	Lipid	mg lipid/g protein	Per cent of total lipid
	Total lipid[b]	300 ± 90	100.0
	Phospholipid[b]	266 ± 30	88.7 ± 0.9
	Nonpolar lipid[b]	34 ± 3	11.5 ± 0.9
0.97	Cholesteryl esters[c]	< 0.9	< 0.5
0.70	Triglycerides[c]	16.8	5.6
0.59	Coenzyme Q[c]	6.9	2.3
0.43	Free fatty acids[c]	4.8	1.6
0.28	Cholesterol[c]	4.2	1.4

[a]Values in solvent II.

[b]Averages for five determinations followed by ± standard deviation.

[c]Averages for two determinations.

4 and ADP/O about 1.8 in succinate and about 2.7 in glutamate).

Table I gives the endogenous oxygen uptakes of washed mitochondria measured by polarography. This uptake is more markedly stimulated by ATP, 87%, than by ADP, 59%, but simultaneous addition of ATP and ADP increases the respiration by 300%.

Figure 1 shows the kinetics of the O_2 uptake as measured by the manometric technique during 30-40 min. In the controls the O_2 uptake reached a constant value after 25 min (Fig. 1a). ATP and NAD^+ additions not only stimulated the respiration but maintained a constant rate for at least 30 min (Fig. 1b, c). The oxygen consumption was also increased by hexokinase + glucose but it approached a constant value after 25 min (Fig. 1d). Calculated P/O ratios are given in Table II.

After 30 min incubation the respiratory level of control mitochondria can still be stimulated by ATP addition but is no longer affected by ADP.

Lipid Composition of Pig Heart Mitochondria

The total lipid and percentages of nonpolar lipids and phospholipids of pig heart mitochondria are shown in Table III. Phospholipids are the main constituents.

Nonpolar Lipids

The TLC of nonpolar lipids in the solvent systems I-IV shows by comparison with standards, the presence of triglycerides, free fatty acids, cholesterol, cholesteryl esters and another component X. Then a small amount of each compound was isolated by preparative TLC in solvent II and analyzed specifically. Triglyceride presence was confirmed by glycerol released after saponification; free fatty acids were identified by their transformation into methyl esters. The Liebermann-Burchard reaction (32) gave a deep green color, specific of unsaturated sterols, with the free cholesterol fraction. Lowry reagent spray (22) stained with marked blue cholesteryl esters and cholesterol on chromatogram. Component X had a Rf value of 0.59 in solvent II; it was purple colored by Rhodamine B and showed a characteristic UV absorption at 275 nm in ethanol; these tests

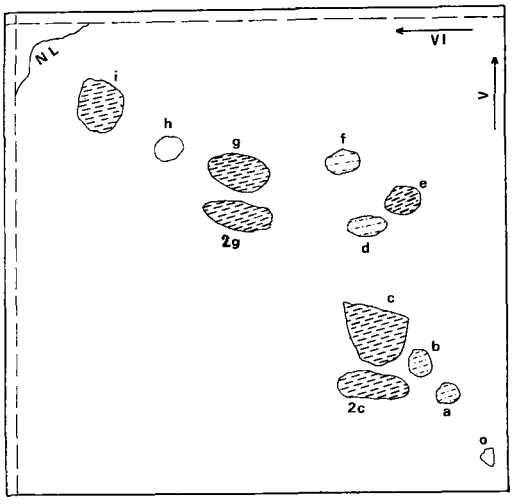

FIG. 2. Two-dimensional TLC of pig heart mitochondria lipids. After development in the first direction with solvent V: chloroform-methanol-water (65:25:4), the lipid track was sprayed with an acid $HgCl_2$ solution and developed in the second direction with solvent VI: butanol-acetic acid-water (60:20:20). Spots identity: o, origin; a, 1-acyl-3-phosphatidylcholine; b, sphingomyelin; c, 3-phosphatidylcholine; 2c, 2-acyl-3-phosphatidylcholine, d, 1-acyl-3-phosphatidylethanolamine; e, 3-phosphatidylinositol; f, 3-phosphatidyl-1'-glycerol; g, 3-phosphatidylethanolamine; 2g, 2-acyl-3-phosphatidylethanolamine; h, unknown; i, cardiolipin, NL, neutral lipids.

TABLE IV

Phospholipid Composition of Pig Heart Mitochondria

Phospholipids[a]	Per cent of total phospholipid
3-Phosphatidylcholine	26.7 ± 1
1-Alkenyl-2-acyl-3-phosphatidylcholine	15.7 ± 0.9
1-Acyl-3-phosphatidylcholine	0.5 ± 0.2
3-Phosphatidylethanolamine	17.2 ± 0.7
1-Alkenyl-2-acyl-3-phosphatidylethanolamine	13.3 ± 0.7
1-Acyl-3-phosphatidylethanolamine	< 0.3
Cardiolipin	18.1 ± 0.5
3-Phosphatidylinositol	4.5 ± 0.2
Sphingomyelin	1.7 ± 0.3
3-Phosphatidyl-1'-glycerol	0.9 ± 0.3
Origin	0.7 ± 0.3
Unknown h	1.4 ± 0.6

[a]Averages for ten determinations followed by ± standard deviation.

indicate component X may be a coenzyme Q (14,35-37). Triglycerides are the main constituents of nonpolar lipids (Table III); amounts of free fatty acids, coenzyme Q and cholesterol were lower than the triglycerides. Cholesteryl esters, present in traces, could not be determined.

Phospholipids

For qualitative and quantitative analysis of phospholipids, a two dimensional thin layer system with a high resolution capacity was necessary. We succeeded in combining Rouser (26) and Broekhuyse (27) systems: migration in a first direction in solvent V, then $HgCl_2$ treatment which transforms acetal phospholipids in 2-acyl phospholipids by releasing 1-alkenyl ether group, and then chromatography in the second direction with solvent VI. Figure 2 shows the lipid pattern obtained after staining with iodine and Table IV gives the percentages of the different phospholipids.

Phosphatides were identified by their Rf values and by specific sprays. Acid hydrolysis, performed on the purified isolated compounds, yielded glycerol (except with compound b), α-glycerophosphate, choline (compounds a, b, c, 2c) ethanolamine (compounds d, g, 2g) and inositol (compound e).

Plasmalogens were shown to be present in the phosphatidyl choline and phosphatidyl ethanolamine fractions which with cardiolipin are the most predominant phospholipids. Phosphatidyl inositol was present in smaller amount than phosphatidyl ethanolamine and phosphatidyl choline. Phosphatidyl glycerol which was identified by the periodate-Schiff reagent (29), and sphingomyelin were minor components. Lysophosphatidyl choline and lysophosphatidyl ethanolamine were detected as traces. An additional unknown compound h was seen on the two dimensional chromatogram. No phosphatidyl serine was detected.

Fatty Acids Composition

The fatty acid composition of total lipid, free fatty acids, triglycerides and total phospholipids of pig heart mitochondria was determined. The percentages of the different fatty acids are given in Table V and representative GLC tracings are shown in Figure 3.

Total lipid fatty acids were oleic, linoleic, palmitoleic, arachidonic and saturated acids with 12, 14, 15, 16, 17 and 18 carbon atoms. No other peak ever followed arachidonic methyl ester on the chromatograms. The distribution of fatty acids in the studied fractions shows some differences.

Total lipid had a high content of polyunsaturated fatty acids. The main constituent was linoleic acid which represented 34% of total fatty acids. Unsaturated fatty acids predominated in phospholipids which contained all the arachidonic acid and most of the linoleic acid. Palmitic acid was evenly distributed in free fatty acids, triglycerides and phospholipids, and oleic acid was chiefly located in triglycerides.

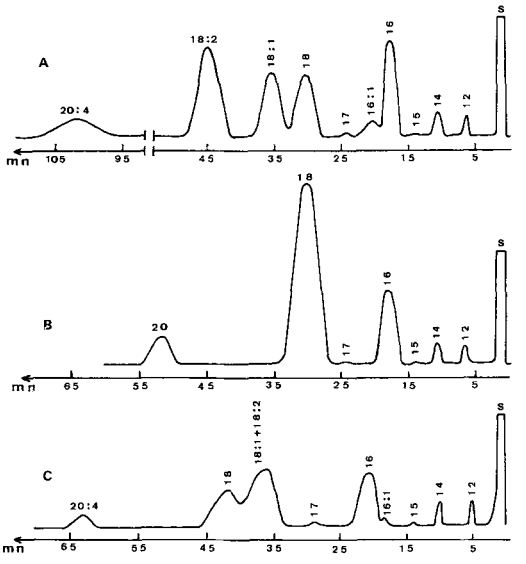

FIG. 3. GLC tracings of the total lipid fatty acid methyl esters. A and B on a 25% DEGS column at 170 C; B, methyl esters were hydrogenated; C, on an Apiezon L column at 210 C; s, solvent peak.

Palmitic and stearic acids were the main saturated fatty acids, present in the three lipid classes; however they predominated in free fatty acids and triglycerides. The remainder, 5% of total lipid fatty acids, consisted of 12, 14 and odd-numbered acids and was particularly located in free fatty acids and triglycerides as were the other saturated acids.

Lipid Variation During Endogenous Respiration

We tried to determine whether endogenous oxygen uptake was supported by lipid degradation. Mitochondria were incubated for 30 min with or without 0.7×10^{-3} M ATP in the absence of added substrate. A systematic study of the lipids was conducted after the incubation on three different preparations.

The total lipids were hardly affected as well as most phospholipids. Yet choline phospholipids including plasmalogens decrease slightly in both conditions. Similarly the levels of lysophosphatidyl choline and free fatty acids increased. Other nonpolar lipids did not vary.

These methods did not allow determination of variations in the saturation of carbon chains.

TABLE V

Fatty Acid Composition of Pig Heart Mitochondria

Fatty acid[a] carbon number	Total lipids[b]	Phospholipids[c]	Triglycerides[c]	Free fatty acids[c]
12	0.5 ± 0.05	---	1	7.3
14	3.3 ± 0.3	1	1.6	6.9
15	1.5 ± 0.4	---	2.1	5
16	14.7 ± 1.2	14.8	32.9	33.4
16:1	4.2 ± 0.9	3.6	5.3	1
17	3.4 ± 0.5	---	1.7	5.5
18	14 ± 0.5	16.6	17.6	25.8
18:1	16.7 ± 0.8	16.3	33.1	13
18:2	33.7 ± 1.2	38.3	3.7	2
18:3	traces	traces	traces	----
20:4	10.8 ± 0.8	9.4	---	---
Σ unsaturated	65.4	67.6	42.1	16
Σ saturated	37.4	32.4	56.9	83.9

[a]In each studied fraction values are expressed in relative percentages of the sum of fatty acids.
[b]Averages for six determinations followed by ± standard deviation.
[c]Averages for three determinations.

DISCUSSION

Endogenous Respiration

In the absence of exogenous ATP, if fatty acids are the main substrates of endogenous respiration they could be easily activated by the intramitochondrial ATP (38). However after 30 min incubation the level values obtained should be due to the fact that ATP and ADP became limiting. This conclusion is supported by the observation that, in the presence of exogenous ATP the respiratory level remains constant. On the contrary, if hexokinase and glucose are added after a preincubation period with ATP, the oxygen uptake is as quick as in latter case for the first 20 min, but then it tends to reach a level value as the ATP becomes limiting for fatty acid activation. Sufficient ATP and ADP concentrations are necessary for efficient fatty acid oxidation.

The variations in lipid composition, although weak, are more than sufficient to account for the observed oxygen uptakes, especially if saturation and desaturation steps are important. The NAD stimulation of the respiratory level is in favor of the fatty acid oxidation (12) as well as the observed P/O ratios of 1. One should remember that the oxidation of exogenous short chain fatty acid by rat liver mitochondria gives a P/O ratio = 1 (39). In the same way, Chefurka (40) has shown that free fatty acids resulting from phospholipid hydrolysis were released during aging of rat liver mitochondria.

Lipid Composition

The main phospholipids of pig heart mitochondria are phosphatidyl choline, phosphatidyl ethanolamine, the corresponding plasmalogens and cardiolipin. Our results present some discrepancy with those of Marinetti et al. (13), who mentioned the presence of phosphatidyl serine and found a greater amount of phosphatidyl inositol. We did not observe phosphatidyl serine in pig heart mitochondria; only a trace, 0.1% or less, of this phospholipid was detected in bovine heart mitochondria (35,36). The high amount of phosphatidyl inositol reported by Marinetti for pig heart mitochondria can be explained by the chromatographic techniques. Indeed Fleischer and Rouser (41), comparing the paper chromatography technique of Marinetti et al. with the two dimensional TLC, found in the beef heart mitochondria greater amounts of phosphatidyl inositol with the Marinetti system. They explained this discrepancy by the decomposition of "altered" form of phosphatidyl ethanolamine (41) and cardiolipin in the paper chromatography.

The fact that Marinetti (13) found no phosphatidyl glycerol as we did, should be related to the insufficient resolution of his solvent system.

Plasmalogens had never been studied in pig heart mitochondria. The distribution we found is similar to those of beef heart (36) and sheep heart (42) mitochondria. Their presence has been interpreted as unnecessary for the electron transfer in beef heart mitochondria (43).

Neutral fats mentioned by Redfearn (14) were identified here to triglycerides and cholesteryl esters only. The amounts of free fatty acids observed in pig heart mitochondria are slightly less than those quoted by Wheeldon et al. in beef heart mitochondria (35).

Up to now nobody had determined pig heart

mitochondria composition of total lipids and distribution of free fatty acids, of phospholipids and triglycerides fatty acids. As compared to beef heart mitochondria (44), we found in total lipids more palmitic acid, threefold more oleic acid and less linoleic acid, and we found heptadecanoic acid. In the triglycerides we found 5% fatty acids $< C_{16}$ not identified by Wheeldon (35) in beef heart mitochondria; besides, these latter mitochondria contain less C_{16}, more C_{18} (saturated and unsaturated) acids. The free fatty acids in pig heart mitochondria are essentially saturated which agrees with values observed in sheep heart (45) but differs from beef heart mitochondria where high levels of polyunsaturated acids were found by Wheeldon et al. (35); but the last authors think that phospholipids are hydrolyzed during fractionation procedures which account for the high level of free polyunsaturated acids. The distribution of phospholipids fatty acids is similar in pig, beef (35,41) or sheep heart mitochondria (45) except for the level of linoleic acid higher in pig heart particles.

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The Synthesis of 1-^{14}C-Arachidonate and 3-^{14}C-Docosa-7,10,13,16-Tetraenoate[1]

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ABSTRACT

Methyl 1-^{14}C-arachidonate was prepared by coupling 1-bromotetradeca-2,5,8-triyne with 4-pentyn-1-ol to yield nonadeca-4,7,10,13-tetrayn-1-ol. This compound was reduced with Lindlars catalyst. The resulting alcohol was converted to the mesylate and then to the nitrile which in turn was converted to methyl 1-^{14}C-arachidonate by hydrolysis with anhydrous HCl in methanol. The methyl 1-^{14}C-arachidonate was reduced to the alcohol with $LiAlH_4$ and converted to the mesylate which in turn was treated with diethyl malonate. Following saponification and decarboxylation 3-^{14}C-docosa-7,10,13,16-tetraenoic acid was obtained.

INTRODUCTION

In studying the metabolism of polyunsaturated fatty acids it is frequently necessary to have available the acid in a radioactive form. In the study reported here the total synthesis of methyl 1-^{14}C-arachidonate and methyl 3-^{14}C-docosa-7,10,13,16-tetraenoate is described.

EXPERIMENTAL PROCEDURES

Synthesis

The methyl 1-^{14}C-arachidonate and the methyl 3-^{14}C-docosa-7,10,13,16-tetraenoate were prepared according to Scheme 1.

1-Bromotetradeca-2,5,8,-triyne (I): The preparation of this compound has been described previously (1).

4-Pentyn-1-ol (II): This compound was prepared by converting tetrahydrofurfuryl alcohol to tetrahydrofurfuryl chloride (2). Tetrahydrofurfuryl chloride was then converted to 4-pentyn-1-ol by treatment with sodium in liquid ammonia (3).

Nonadeca-4,7,10,13-tetrayn-1-ol (III): Compounds I and II were coupled in the usual way (4). The product (28% yield; mp, 46-48C) was recovered by extraction with petroleum ether (1).

Nonadeca-4,7,10,13-tetraen-1-ol (IV): The reduction of 7.9 g of III was carried out with 3 g of Lindlars catalyst (5) in 250 ml of absolute ethanol containing 4 ml of a 5% solution of synthetic quinoline in absolute ethanol at atmospheric pressure. Thin layer chromatography (TLC) of the product on Silica Gel G plates in the solvent system petroleum ether-ether-acetic acid 80:20:2(v/v) showed a major component which had an identical R_f with authentic linoleyl alcohol. Several other minor components were also present. The desired product was recovered by eluting a column of silicic acid with petroleum ether-ether 80:20 (v/v). The effluent was monitored by TLC.

Mesylate of nonadeca-4,7,10,13-tetraen-1-ol (V): The mesylate was prepared essentially by the procedure of Baumann and Mangold (6). To 4 ml of pyridine, cooled in an icebath, was added 1.22 g (4.44 mmoles) of IV followed by 6.66 mmoles of freshly distilled methane sulfonyl chloride. After the reaction mixture had stirred for 5 hr in an ice bath the mesylate was isolated (6) and used without further purification.

1-^{14} Cyano-4,7,10,13-nonadecatetraene (VI): To 4 ml of dry dimethyl sulfoxide (DMSO) containing (V) was added 2.0 mC of Na^{14}CN Amersham Searle; specific activity 52.8 mC/mmole) and 143 mg (2.96 mmoles) of carrier NaCN in 10 ml of DMSO. The reaction mixture was heated for 3 hr at 85-90 C (7). The nitrile was extracted with five 50 ml portions of ether. The pooled ether extracts were washed several times with H_2O and the ether layer was dried over Na_2SO_4. The ether was removed under reduced pressure.

Methyl 1-^{14}C-arachidonate (VII): The nitrile was dissolved in 50 ml of 25% anhydrous HCl in MeOH to which was added 3 mmoles of H_2O. The mixture was stirred for 1 hr under N_2 in an ice bath and then allowed to stand overnight at room temperature. Then the reaction mixture was poured into 100 ml of H_2O and the product was recovered by extraction with petroleum ether. TLC on Silica Gel G plates revealed several components in addition to the major component which had an identical R_f with methyl arachidonate. The entire product (0.762 g) was then streaked on 12 Silica Gel G plates (0.5 mm) which were developed in

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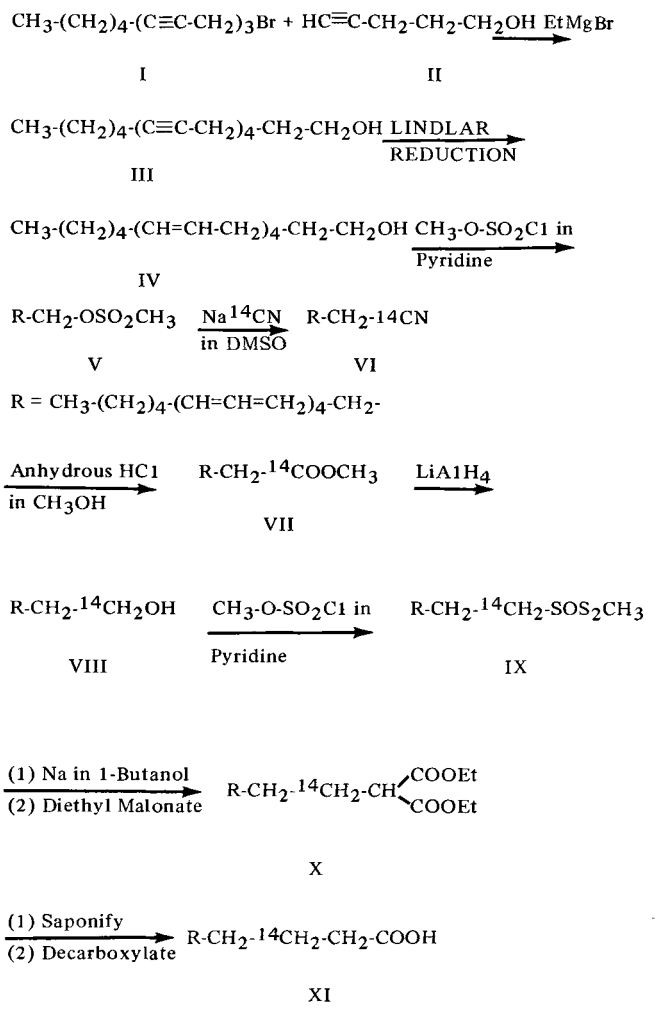

Scheme 1

petroleum ether-ether-acetic acid 80:20:1 (v/v). A column of Unisil (Clarkson Chemical Co., Williamsport, Pa.) was prepared using petroleum ether-ether 70:30(v/v). The TLC plates were sprayed with a 0.2% ethanolic solution of 2',7'-dichlorofluorscein and the component corresponding to authentic methyl arachidonate was scraped from the plates, poured on the column and the product (0.604 g; 67% yield based on NaCN) was recovered by elution with petroleum ether-ether 70:30(v/v).

Although compound VII migrated as a single component when examined by TLC, the *trans* isomers as well as over reduced products would not be eliminated by this method of purification. Accordingly, $AgNO_3$ plates (8) were used to eliminate the *trans* isomers as well as the small amounts of monoenoic, dienoic and trienoic methyl esters which were formed following Lindlar reduction of III. The plates were developed in chloroform-ethanol 97:3(v/v) and the component migrating with authentic methyl arachidonate was scraped from the plate and poured into a chromatographic column containing Unisil. Of the 450 mg of VII applied to the $AgNO_3$ TLC plates 300 mg was recovered by eluting the column with petroleum ether-ether 1:1(v/v).

1-^{14}C-eicosa-5,8,11,14-tetraene-1-ol (VIII): To 70 ml of anhydrous ether was added 1.0 g of $LiA1H_4$ followed by 248 mg of methyl 1-^{14}C-arachidonate in 30 ml of ether. The mixture was stirred for 2 hr at room temperature and then heated at reflux for an additional 2 hr. The alcohol was isolated and purified by Silica Gel G TLC with petroleum

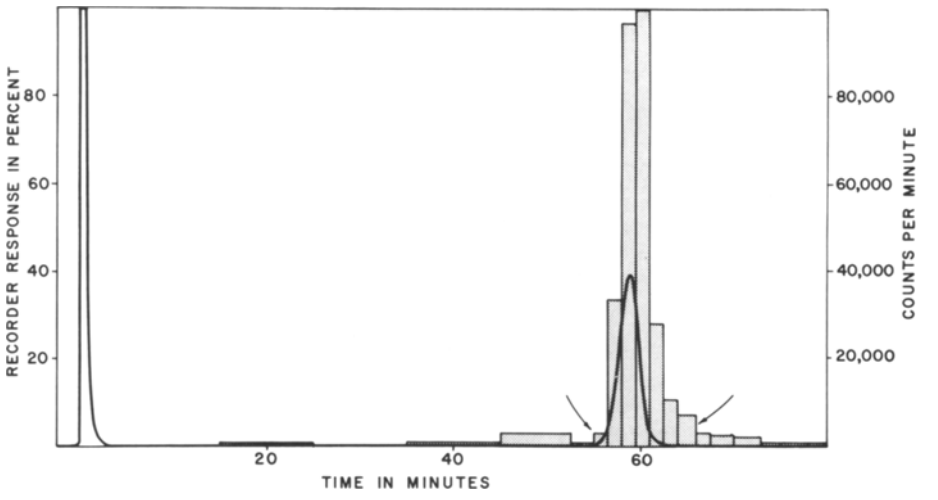

FIG. 1. Gas liquid chromatogram of methyl 1-^{14}C-arachidonate.

ether-ether-acetic acid 80:20:1(v/v). The product (184 mg; 81% yield) was recovered by scraping the alcohol band from the plate, transferring it to a column of Unisil and eluting with ether.

Mesylate of 1-^{14}C-eicosa-5,8,11,14-tetraen-1-ol (IX): The mesylate was prepared as described for V.

Diethyl 1-^{14}C-eicosa-5,8,11,14-tetraenyl malonate (X): To 5 ml of 1-butanol was added 75 mg (3.21 mmoles) of sodium. When the sodium had dissolved, 4.5 mmoles of diethyl malonate were added in 5 ml of 1-butanol. The mixture was heated to reflux and the mesylate (IX) was added in 6 ml of 1-butanol. The reaction mixture was heated at reflux for 3 hr.

Methyl 3-^{14}C-docosa-7,10,13,16-tetraenoic acid (XI): The 1-buanol was removed from the above reaction mixture and 50 ml of a 4% solution of KOH in EtOH-H_2O 9:1(v/v) was added. The compound (X) was saponified by stirring overnight under N_2. Then the reaction mixture was acidified and extracted with ether. The ether was removed under reduced pressure and 10 ml of synthetic quinoline was added. The compound was decarboxylated by heating it at 130-140 C for 3 hr in an oil bath under N_2. The reaction mixture was acidified with 3 N HCl and extracted with ether. The acid was purified by preparative TLC and recovered by eluting the fatty acid from the silica gel with petroleum ether-ether 80:20(v/v). The acid was converted to the methyl ester by stirring overnight with a 5% solution of anhydrous HCl in MeOH. The methyl ester was purified by $AgNO_3$ TLC as described for VII. The final yield was 97 mg (44% based on the alcohol [VIII] used).

Thin Layer Chromatography

The radioactive and chemical purity of the methyl esters was determined by TLC using the solvent system petroleum ether-ether-acetic acid 90:10:1(v/v). The plates were stained in an iodine chamber and 1 cm bands of silica gel were scraped from the plates into scintillation vials and counted in a liquid scintillation spectrometer using 10 ml of the water-dioxane medium described by Synder (9). Counts per minute (cpm) were converted to disintegrations per minute (DPM) by use of an applied external standard.

The solvent system chloroform-ethanol 97:3(v/v) was used for $AgNO_3$ TLC. After development the plates were sprayed with 2',7'-dichlorofluorscein and bands of the silica gel were transferred to small columns contain-

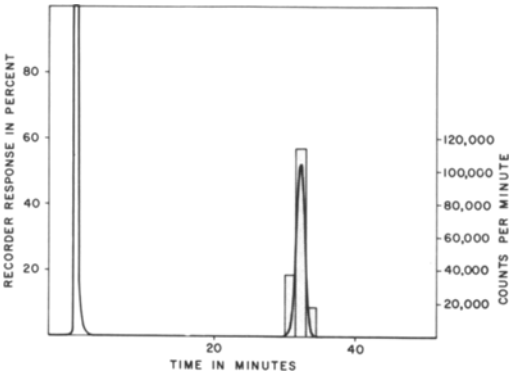

FIG. 2. Gas liquid chromatogram of methyl 1-^{14}C-arachidate mixed with authentic methyl arachidate.

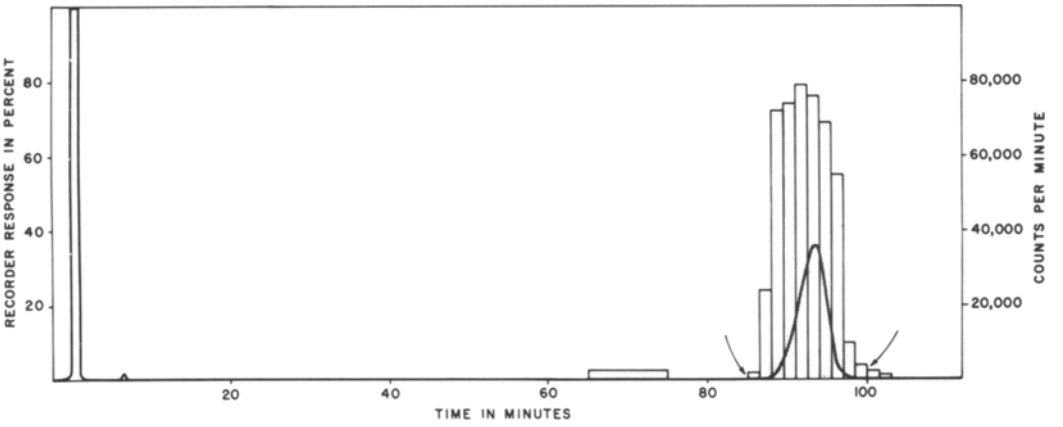

FIG. 3. Gas liquid chromatogram of methyl 3-^{14}C-docosa-7,10,13,16 tetraenoate.

ing Unisil. The columns were eluted with petroleum ether-ether 70:30(v/v) and the effluent was taken to dryness and radioactivity was measured as described above.

Gas Liquid Chromatography

Gas liquid chromatography (GLC) was carried out on an F and M Model 810 gas chromatograph equipped with a thermal conductivity detector. The stainless steel columns, 10 ft long by 1/4 in. diameter, were packed with 15% ethylene glycol succinate on 80-100 mesh Gas-Chrom P. The oven temperature was maintained at 195 C while both the injector and detector were 280 C. The flow rate of helium was 60 ml/min. Fractions were collected from the thermal conductivity detector in glass cartridges (Packard) containing two cellulose filters (Packard) using a Packard fraction collector. The cartridges were transferred to scintillation vials and 10 ml of scintillation fluid was added. The scintillation fluid contained 5.0 g of 2,5-diphenyloxazole and 0.3 g of 1,4-bis [2-(4-methyl-5-phenyloxazoyl)]-benzene per liter of toluene.

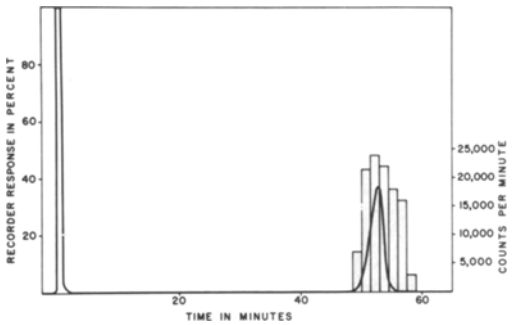

FIG. 4. Gas liquid chromatogram of methyl 3-^{14}C-docosanoate mixed with authentic methyl docosanoate.

Infrared Spectra

Infrared spectra were run as smears on NaCl infrared cells using a Perkin Elmer Model infrared 337 spectrophotometer.

RESULTS

Methyl 1-^{14}C-arachidonate and methyl 3-^{14}C-docosa-7,10,13,16-tetraenoate migrated as single components together with methyl arachidonate on TLC. It was found that 99.6% of the recovered dpm in methyl 1-^{14}C-arachidonate were associated with the component migrating with authentic methyl arachidonate. With methyl 3-^{14}C-docosa-7,10,13,16-tetraenoate 99.5% of the dpm were associated with the component migrating with authentic methyl arachidonate. With $AgNO_3$ TLC of methyl 1-^{14}C-arachidonate 97.8% of the recovered dpm were associated with a component that migrated together with methyl arachidonate. For 3-^{14}C-docosa-7,10,13,16-tetraenoate 99.3% of the recovered dpm were associated with a component that migrated together with methyl arachidonate. In the above four analyses the recovery of radioactivity was between 88-95% of that which was applied to the TLC plates.

Figure 1 shows a gas chromatogram of methyl 1-^{14}C-arachidonate. The radioactivity included by the arrows in Figure 1 represents a purity of 96%. The small radioactive contaminant appearing before methyl arachidonate is probably a methyl ester of eicosatrienoic acid which was not completely eliminated by

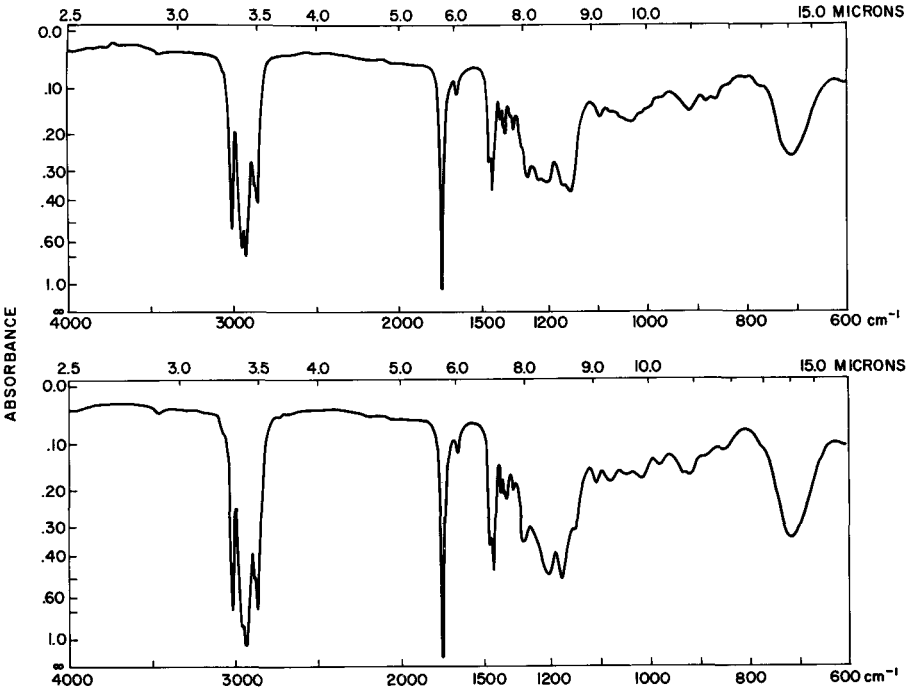

FIG. 5. Infrared spectra of methyl 1-^{14}C-arachidonate (top) and methyl 3-^{14}C-docosa-7,10,13,16-tetraenoate (bottom).

$AgNO_3$ TLC.

When a portion of methyl 1-^{14}C-arachidonate was reduced with platinum oxide and mixed with authentic methyl arachidate and analyzed by GLC a single component was obtained which contained 98% of the recovered radioactivity (Fig. 2).

Figure 3 shows a gas chromatogram of methyl 3-^{14}C-docosa-7,10,13,16-tetraenoate. The radioactivity included by the arrows represents a purity of 96%. Again the small radioactive component appearing before methyl docosa-7,10,13,16-tetraenoate is probably a methyl ester of docosatrienoic acid which was not removed by $AgNO_3$ TLC.

When a portion of methyl 3-^{14}C-docosa-7,10,13,16-tetraenoate was reduced and mixed with authentic methyl docosanoate a single component was found (Fig. 4). Of the recovered radioactivity 98% was associated with this component.

In the above four analyses, the recovery of radioactivity from the gas chromatograph varied from 90-99% of that which was injected.

Figure 5 depicts the infrared spectra of methyl 1-^{14}C-arachidonate and methyl 3-^{14}C-docosa-7,10,13,16-tetraenoate. Both methyl esters were essentially free of *trans* double bonds as measured by the absorption at 965 cm^{-1}.

The methyl 1-^{14}C-arachidonate and the methyl 3-^{14}C-docosa-7,10-13,16-tetraenoate had specific activities of 0.40 mC/mmole.

DISCUSSION

It has been a consistent observation (10,11) that commercially available Na^{14}CN contains substantial amounts of radioactivity in some other form (possibly $^{14}CO_3$=). For this reason the specific activity of the products is not identical with that of the precursor although their purity is not affected.

Stoffel (12) has prepared 1-^{14}C-arachidonic acid by coupling 1-bromo-tetradeca-2,5,8-triyne with 1-chloro-4-pentyne to yield 1-chlorononadeca-4,7,10,13-tetrayne. This compound has a melting point of 18-20 C and polymerizes rapidly in the presence of light and air. Our procedure differs in that the nonadeca-4,7,10,13-tetrayn-1-ol has a melting point of 46-48 C and is quite stable under atmospheric conditions. The compound can be stored under nitrogen at -20 C for prolonged periods of time without any apparent decomposition.

Klenk and Mohrhauer (13) have described a procedure for the chain extension of acids in which the methyl ester is reduced with $LiAlH_4$

to the alcohol. The alcohol is then converted to the tosylate and then to the iodo-derivative. The iodo-derivative is then treated with diethyl malonate which after saponification and decarboxylation yields the desired acid. Recently Marcel and Holman (11) have shown that a mesylate can be treated with the sodium salt of diethyl malonate. Following saponification and decarboxylation the desired acid was obtained. This latter approach has the advantage in that a minimum number of steps is required in preparing 3-^{14}C-acids. In the study reported here it has been established that this general procedure is satisfactory for the preparation of 3-^{14}C-acids containing four double bonds.

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Lipid Metabolism of Acetate-1-^{14}C by Leukocytes From Dogs Fed an Arteriosclerosis-Inducing Diet

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ABSTRACT

Dogs were maintained on a control ration or on a semisynthetic diet, containing 5% cholesterol and 16% hydrogenated coconut oil, known to induce hyperlipemia and arteriosclerosis. Circulating leukocytes isolated from dogs fed the coconut oil containing diet were shown to incorporate 50% more radioactivity into lipids than control leukocytes when incubated with acetate-1-^{14}C. This increase, expressed as dpm/mg of leukocyte DNA, was not specific for any particular lipid since the distribution of radioactivity between neutral lipids and phospholipids, as well as among their subfractions, was the same regardless of the diet fed. The labeling patterns observed suggest that leukocyte fatty acid synthesis from acetate occurs predominantly, and perhaps exclusively, by the chain elongation pathway.

INTRODUCTION

Although several investigators have been concerned with the question of whether lipid-laden leukocytes or lipophages are involved in atherogenesis, and some have shown an effect of diet on lipemia and on the lipid content of circulating leukocytes (1-4), little attention has been devoted to the relationship between diet, plasma lipemia and leukocyte lipid synthesis. The purpose of this study was to determine whether lipid metabolism in blood leukocytes would be influenced by feeding a diet known to induce hyperlipemia and arteriosclerosis in dogs. Furthermore, if changes were observed, subsequent studies could be planned to ascertain whether they are characteristic or indicative of the type of abnormal lipid metabolism contributing to foam cell formation and lipid accumulation in the arterial wall.

METHODS

Adult male mongrel dogs weighing 12-20 kg were maintained on control ration (5) or a semisynthetic diet lacking essential fatty acids (Diet I), which has been shown to induce arteriosclerosis when fed for 1 year or longer (5-7). The composition and fatty acid distribution of Diet I, which contained 5% cholesterol and 16% hydrogenated coconut oil as the only source of lipid, was described previously (5). Blood leukocytes were isolated from dogs kept on these diets for 2-16 months.

Venous blood obtained from animals fasted for at least 16 hr was mixed with commercially prepared Acid Citrate Dextrose solution (ACD) (U.S.P. formula B, Abbott Laboratories, North Chicago, Ill.) containing 1.47 g glucose, 1.32 g sodium citrate and 0.44 g anhydrous citric acid per 100 ml solution, in a ratio of 4 volumes blood to 1 volume ACD. This mixture served as the source of leukocytes (WBC) which were isolated as described earlier (8) by a method involving sedimentation of erythrocytes with an equal volume of 3% high molecular weight dextran in 0.9% NaCl, centrifuging the remaining plasma-WBC suspension at 110 x g and washing the collected leukocytes twice with 15 volumes of 0.9% NaCl containing 1 mg glucose/ml. Sedimented erythrocytes were also washed and resuspended in 0.9% NaCl (8). Leukocytes isolated from 80-100 ml of citrated blood were resuspended to 10 ml with the isotonic saline plus glucose solution. Autologous plasma was then added to give a total volume of 21 ml from which 3 ml of the WBC suspension were removed for determination of DNA, cell volume and cell counts.

Labeled sodium acetate-1-^{14}C, obtained from Amersham/Searle, Chicago, Ill., with a specific activity of 44.4 mc/mmole, was dissolved in 0.9% NaCl to give a concentration of 50 μc/ml. The acetate which had a stated purity of 98% was found by strip scanning to give only one radioactive spot after chromatoraphy on Whatman No. 1 paper in ethanol-ammonium hydroxide-water 80:4:16. Incubation of the leukocyte suspension was begun after adding 2 ml of labeled acetate to give a final volume of 20 ml. The 20 ml mixture thus contained 100 μc and 2.26 μmoles of acetate-1-^{14}C, 9.43 ml of plasma and 8.57 ml of WBC suspended in saline-glucose. Incubations were carried out in capped 125 ml Erlenmeyer flasks fitted with rubber serum stoppers, through which 3 ml Hyamine hydroxide were injected into a glass vial inside the

TABLE I

Leukocyte Neutral Lipids

Neutral lipid	(dpm/mg leukocyte DNA) Control (N=8)	Diet I (N=10)	p<
Free cholesterol[a]	17,420 ± 2,142[b]	18,679 ± 840	n.s.
Free fatty acid	103,742 ± 7,939	165,829 ± 11,977	0.001
Triglyceride	79,979 ± 7,097	116,186 ± 12,403	0.05
Cholesteryl ester	12,118 ± 980	19,926 ± 2,158	0.01
Total	213,259,± 12,004	320,620 ± 18,866	0.001

[a]Free cholesterol fraction may also contain diglycerides.
[b]Disintegrations per minute (dpm) ± standard error.

flask after 2 hr of gentle shaking at 37 C. After an additional 0.5 hr incubation the flasks were removed, placed in an ice bath and 20 ml methanol were added. The entire leukocyte plus plasma suspension was then extracted with chloroform-methanol 2:1 (9). Zero time studies in which methanol was added to the incubation mixture preceding the addition of labeled acetate showed that 99.9% of the radioactivity was found in the aqueous phase. The subsequent wash of the organic phase with 0.2 volume of 0.02% $CaCl_2$, which was done in all extractions, removed virtually all of the remaining labeled material from the organic phase.

Neutral lipids and phospholipids were separated on silicic acid columns by eluting with chloroform and methanol, respectively, and further fractionated by thin layer chromatography (TLC) on Silica Gel G. Neutral lipids were divided into bands containing free cholesterol probably contaminated with diglyceride, free fatty acid, triglyceride and cholesteryl ester by developing with petroleum ether-diethyl ether-acetic acid 90:10:1. Plates streaked with the phospholipid fraction were developed with the chloroform-methanol-acetic acid-water 25:15:4:2 system of Skipski et al. (10) yielding lysophosphatidyl choline, sphingomyelin, phosphatidyl choline, phosphatidyl serine plus phosphatidyl inositol, phosphatidyl ethanolamine and near the solvent front, phosphatidic acid plus cardiolipin. Bands were visualized by exposing the plates to iodine vapor. Radioactivity in each fraction was measured by scraping the silica gel from the TLC plate and transferring it directly to scintillation vials containing naphthalene, PPO, dimethyl POPOP, dioxane and water as described by Snyder (11). Disintegrations per minute (dpm) were calculated by external standardization. Recovery of radioactivity in terms of per cent of the total dpm applied to the TLC plates was always greater than 93%. Amounts of radioactivity detected in various lipid fractions were expressed as dpm/mg of lipid or mg of DNA, since accurate leukocyte counts were difficult to obtain from the WBC suspensions. DNA was measured by the Webb and Levy method (12).

Fatty acid methyl esters were prepared from lipid fractions isolated by column chromatog-

TABLE II

Leukocyte Phospholipids

Phospholipid	(dpm/mg leukocyte DNA) Control, (N=7)	Diet I (N=9)	p<
Origin	511 ± 170	1,558 ± 291	0.01
Lysophosphatidyl choline	1,222 ± 201	3,604 ± 742	0.025
Sphingomyelin	19,992 ± 1,783	28,110 ± 2,451	0.025
Phosphatidyl choline	84,123 ± 8,255	117,292 ± 9,400	0.025
Phosphatidyl serine + phosphatidyl inositol	16,505 ± 1,987	26,457 ± 682	0.001
Phosphatidyl ethanolamine	26,358 ± 2,448	30,278 ± 1,211	n.s.
Phosphatidic acid + cardiolipin	53,114 ± 5,105	86,765 ± 8,872	0.01
Total	201,114 ± 14,854	294,064 ± 19,850	0.005

TABLE III

Distribution of Radioactivity

Lipid fraction	Control, %		Diet I, %	
Free cholesterol	4.20		3.04	
Free fatty acid	24.99		26.98	
Triglyceride	19.27		18.90	
Cholesteryl ester	2.92		3.24	
Total neutral lipid	51.38	213,259 dpm[a]	52.16	320,620 dpm
Origin	0.12		0.25	
Lysophosphatidyl choline	0.29		0.59	
Sphingomyelin	4.81		4.57	
Phosphatidyl choline	20.27		19.08	
Phosphatidyl serine + phosphatidyl inositol	3.98		4.30	
Phosphatidyl ethanolamine	6.35		4.93	
Phosphatidic acid + cardiolipin	12.80		14.12	
Total phospholipid	48.62	201,825 dpm	47.84	294,064 dpm
Total lipid		415,084 dpm		614,684 dpm

[a]Disintegrations per min/mg leukocyte DNA.

raphy or TLC using boron trifluoride in methanol (13). Whenever fatty acid analysis was determined on fractions separated by TLC, the lipids were detected by fluorescence in UV light after spraying the plate with 0.04% dichlorofluorescein in 50% ethanol.

Separation was achieved by utilizing a 5000 Series Barber-Colman gas chromatograph equipped with a 6 ft x 3.5 mm ID coiled glass column and flame ionization detector. Column packing was 6% stabilized ethylene glycol adipate on high performance 80/100 mesh Chromosorb G. Carrier gas was helium or nitrogen at 40 ml/min and the column was operated isothermally at 200 C. Distribution of the label among fatty acids of lipid fractions was estimated with the Model 5190 Radioactivity Monitoring System attachment. By means of a stream splitter, 90% of the column effluent was diverted to a 650 C combustion tube containing CuO which converted the methyl esters to labeled carbon dioxide which was then counted and recorded separately but simultenaously with the mass. Distribution of both fatty acid mass and radioactivity were calculated by the peak height times retention time method which was found not to differ significantly from the area percentage method obtained with the disc integrator (14). Recorded responses of the proportional counter to injections of equal amounts of radioactivity were identical regardless of whether labeled palmitate, stearate or oleate methyl esters were used. Calculation of the specific activity of each labeled fatty acid would have been possible with the system but was not done in these experiments, because such results would have little value since the extracts used contained not only WBC fatty acids but considerably greater and varying amounts of fatty acids contributed by the autologous plasma present in the original incubation mixture.

Blood smears were prepared for differential counting and to assess the extent of lipid accumulation and vacuole formation. Smears were fixed in 4 parts Earle's balanced salt solution and 1 part 40% formaldehyde for 10 min, rinsed three times in distilled water, dehydrated in propylene glycol, stained with Sudan IV or Oil Red O solutions for 5 min, rinsed twice in 70% ethanol, counter-stained with 50% May-Grünwald solution for 1 min, rinsed twice with distilled water and dried in air.

RESULTS

Changes in the metabolism of acetate-1-^{14}C by leukocytes isolated from dogs on atherogenic Diet I seemed to arise relatively rapidly and probably coincided with the rapidly changing plasma lipid pattern described elsewhere (5). Incubations carried out after animals had been on the diet 2-16 months gave similar results and did not appear to be related to length of time on either diet. Results from all dogs on a particular diet were therefore averaged and treated as a single group. Differences in calculated means were statistically evaluated by applying Student's t test. The incorporation

TABLE IV

Distribution of Radioactivity Among Fatty Acids

	Neutral lipid, %		Phospholipid, %	
Fatty acid	Control	Diet I	Control	Diet I
16:0	13.6	12.3	5.7	5.1
16:1	2.1	1.9	0.8	1.0
18:0	30.0	23.9	15.9	13.8
18:1	5.1	7.9	6.8	7.9
20:1[a]	19.1	15.5	14.4	18.6
20:2[a]	15.2	15.4	9.9	15.0
>20:2	15.0	23.1[b]	46.6	38.8

[a]Tentative identification was aided by prior separation of the methyl esters of thin layer chromatography plates impregnated with silver ion (26).

[b]Differences between the Control group (N=3) and Diet I group (N=6) were not significant except for the radioactivity associated with fatty acids leaving the column after C 20:2 in the neutral lipid fraction ($p < 0.05$).

of label into neutral lipids is expressed in Table I as dpm/mg of leukocyte DNA. Table II depicts the pattern found in the phospholipids. Radioactivity in the total lipid was approximately equally distributed between neutral lipid and phospholipid regardless of which diet the dogs were fed as seen in Table III. Also shown in the same table is the percentage of the total dpm each neutral lipid and phospholipid subfraction contained. Similarities in distribution percentages strongly suggest that the biosynthesis of lipids in leukocytes, as judged by incorporation of labeled acetate into various lipid fractions, proceeds by the same pathways in WBC from dogs on Diet I as from controls. These similarities between groups do not extend, however, to the total amount of radioactivity incorporated which in nearly every lipid subfraction was significantly higher in the Diet I group expressed as dpm/mg leukocyte DNA (Tables I, II and III).

Not included in the tables are data obtained from leukocytes isolated from dogs fed Diet I but without the cholesterol supplement for 3 months. Only three animals were studied but incubation with labeled acetate gave results indistinguishable from those given for the group fed Diet I.

Most of the radioactivity in lipids was found in the long chain fatty acid moieties. Distribution of the label among the lipid classes was not significantly influenced by the diet fed (Table III), although there were striking differences between neutral lipid and phospholipid fatty acid labeling as shown in Table IV. Fatty acid patterns in isolated and washed leukocytes which were not mixed with plasma or isotope are given in Table V. In spite of the altered plasma fatty acid composition (5) and the lipid vacuolization observed in some WBC as described below, no differences were noted in the neutral lipid fatty acids. Only in phospholipids were there significant decreases in linoleic and arachidonic acids and an increase in the eicosatrienoic acids characteristic of essential fatty acid deficiency.

Examination of blood smears revealed that all dogs on Diet I had lipid-laden circulating cells (polymorphonuclear leukocytes, mono-

TABLE V

Leukocyte Fatty Acid Distribution[a]

	Neutral lipid, %		Phospholipid, %		
Fatty acid	Control	Diet I	Control	Diet I	
12:0	2.0	2.7	0.7	1.8	
12:1	0.1	0	0	0.1	
14:0	8.8	8.9	2.5	4.9	($p < 0.05$)
14:1	2.6	1.7	0.1	0.1	
14:1 - 16:0	6.8	3.9	0.9	1.0	
16:0	25.1	27.7	20.6	22.8	
16:1	15.7	15.0	3.6	4.9	
16:1 - 18:0	2.9	2.6	0.8	0.5	
18:0	10.0	12.9	17.3	16.1	
18:1	22.2	21.2	11.4	15.6	
18:2	3.8	2.8	6.0	4.5	($p < 0.025$)
>18:2	0	0.3			
18:2 - 20:3			4.7	4.1	
20:3 w9			0.4	3.9	($p < 0.05$)
20:3 w6			0.6	1.4	($p < 0.05$)
20:4			17.2	8.3	($p < 0.01$)
>20:4			13.6	9.7	

[a]Lipids were extracted from leukocytes not subjected to incubation or addition of acetate. Control group (N=2). Diet I group (N=4).

cytes and lymphocytes) 10 days to 2 weeks after initiation of the diet. At this time, electron microscopy demonstrated membrane-bound osmiophilic intracellular vacuoles as well as lipid accumulation on the surface of the cell membrane. Periodic sampling of fasting peripheral blood showed that extracellular lipid coats appeared during the first 6 days following initiation of the diet and intracellular lipid vacuoles 4-8 days thereafter. Differential counts of peripheral blood smears and buffy coats failed to demonstrate any correlation between distribution of leukocyte populations and severity of established hyperlipemia. Quantitative data on the total lipid content of leukocytes unfortunately were not obtained, since WBC were not isolated from the incubation mixture prior to lipid extraction and subtraction of that portion owing to plasma lipid did not give reliable values for the WBC contribution to the total. Extraction of washed red cells from dogs on Diet I showed no increase in total lipid weight per cell or per gram of hemoglobin even after several months on the diet.

While the present study is focused on the lipid metabolism of acetate, it should be noted that only between 1% and 2.5% of the labeled acetate was incorporated into extracted lipid at the end of the 2.5 hr incubation whereas 30-60% of the ^{14}C was found trapped as $^{14}CO_2$ with the remainder in the aqueous phases, probably largely as unreacted carboxyl labeled acetate. Variability in the recovery of labeled carbon dioxide prevented any meaningful comparison between groups in their capacity to oxidize labeled acetate.

DISCUSSION

Leukocytes isolated from dogs fed a diet known to induce arteriosclerosis incorporated approximately 50% more radioactivity from acetate-1-^{14}C into both neutral lipids and phospholipids. The increase was not specific for any lipid fraction since the distribution of radioactivity was the same in control WBC lipids as in those from the Diet I group. Dietary cholesterol does not seem to be a determining factor in acetate utilization since leukocytes from three dogs fed a diet identical to Diet I but without the cholesterol supplement for three months gave the same results as those obtained from the group fed Diet I. Although incubations were done on mixed leukocyte populations, the increase cannot be attributed to differences in types of WBC isolated since the differential counts in both whole blood and in the incubation suspensions were the same as for control preparations. That it was related to intracellular lipid content cannot be established since leukocyte total lipid weight was not obtained and, though there were indications by cytochemical staining that some WBC contained lipid vacuoles, fatty acid distribution of leukocyte neutral lipids did not show any deviation from control values and phospholipids were not substantially altered in spite of quite marked changes in plasma lipid fatty acid patterns.

Incubations were carried out in the presence of autologous plasma in order to approximate more closely the in vivo condition than if buffer or saline alone were used. Labeled free fatty acids are known to be transported out of leukocytes into plasma at a rapid rate in vitro when rabbit blood is incubated with acetate-2-^{14}C (15). Lipids newly synthesized by human WBC and platelets are also transferred out of cells into plasma (16). Transport of free fatty acids across platelet membranes was shown to require the presence of plasma since no labeled lipid was released into the medium when platelets were incubated with acetate in buffer alone (17,18). Whether increased acetate conversion to lipid in WBC from Diet I dogs is an acquired property of the leukocytes themselves or is mediated by plasma lipid composition or other plasma factors will be investigated. Conditions reported to have an effect on acetate metabolism by circulating leukocytes include 4-7 hr postalimentary lipemia (19), phagocytosis (20) and leukemia (21). Sen and Roy (22) showed an increase in incorporation of radioactivity into WBC lipids when whole blood from hypertensive patients was incubated with acetate-1-^{14}C. Another aspect to consider in evaluating the incorporation of label into the cholesteryl ester fraction when incubations are carried out in plasma is that labeled free cholesterol or lecithin could serve as the precursor in plasma for cholesteryl ester synthesis by the action of lecithin-cholesterol acyltransferase.

Distribution of radioactivity among fatty acids of both neutral and phospholipids demonstrated that all of the labeled acetate appeared in long chain fatty acids, the shortest of which was palmitic (C 16:0). Fatty acids may be synthesized by more than one pathway. The de novo pathway commonly found in the cell sap of many tissues involves a carboxylation reaction whereby acetyl coenzyme A reacts with CO_2 and ATP to yield malonyl-CoA and is catalyzed by acetyl-CoA carboxylase. Palmitic acid is the end product of the reaction that utilizes 1 acetyl-CoA, 7 malonyl-CoA and 14 NADPH, and is catalyzed by the multienzyme complex called fatty acid synthetase. Many

investigators have used the appearance of acetate label in C 16:0 as evidence for the existence of the de novo pathway, and the finding of radioactivity in stearate (C 18:0) or longer chain acids as proof of the "mitochondrial" chain elongation mechanism in which successive acetyl units are added on to preexisting long chain fatty acids without going through malonyl-CoA formation. Miras et al. (23), however, found that in human leukocytes incubated with acetate-1-^{14}C the small amount of radioactivity detected in C 16:0 was formed mainly through the elongation pathway since most of the label was located in the carboxyl group. Majerus and Lastra (24) have shown furthermore that neither human leukocytes nor mature erythrocytes are capable of de novo fatty acid synthesis because they lack acetyl-CoA carboxylase activity. However platelets do contain the complete de novo system including carboxylase (25).

It thus appears that while the data presented concerning fatty acid synthesis do not prove that the de novo pathway is not involved to some extent, the results strongly indicate that the chain elongation pathway is the predominant system, and perhaps the only one, operative in circulating leukocytes.

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The Structures of the Branched Fatty Acids in the Wax Esters of Vernix Caseosa

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ABSTRACT

By combined gas liquid chromatography-mass spectrometry a series of monomethyl branched fatty acids was found in the fatty acid moiety of the wax esters of vernix caseosa. The methyl branch occurred on the even C-atoms of chains ranging from C_{11} to C_{17} (some 43 compounds in all). Except for the iso acids and possibly some of the anteiso acids, these could be formed by replacement of malonyl CoA with a molecule of methyl malonyl CoA at the point of the branch. Smaller amounts of fatty acids also were found with two methyl branches occurring on the even C-atoms of chains ranging from C_9 to C_{15}.

INTRODUCTION

We have previously reported the presence of five series of branched fatty acids in vernix caseosa and in human skin surface lipids, largely on the basis of gas liquid chromatography (GLC) (1). By means of combined GLC-mass spectrometry we have now found these to be positional isomers of monomethyl and dimethyl branched acids.

EXPERIMENTAL PROCEDURE

Vernix caseosa, the greasy material on the skin of the newborn, was used as sample of skin

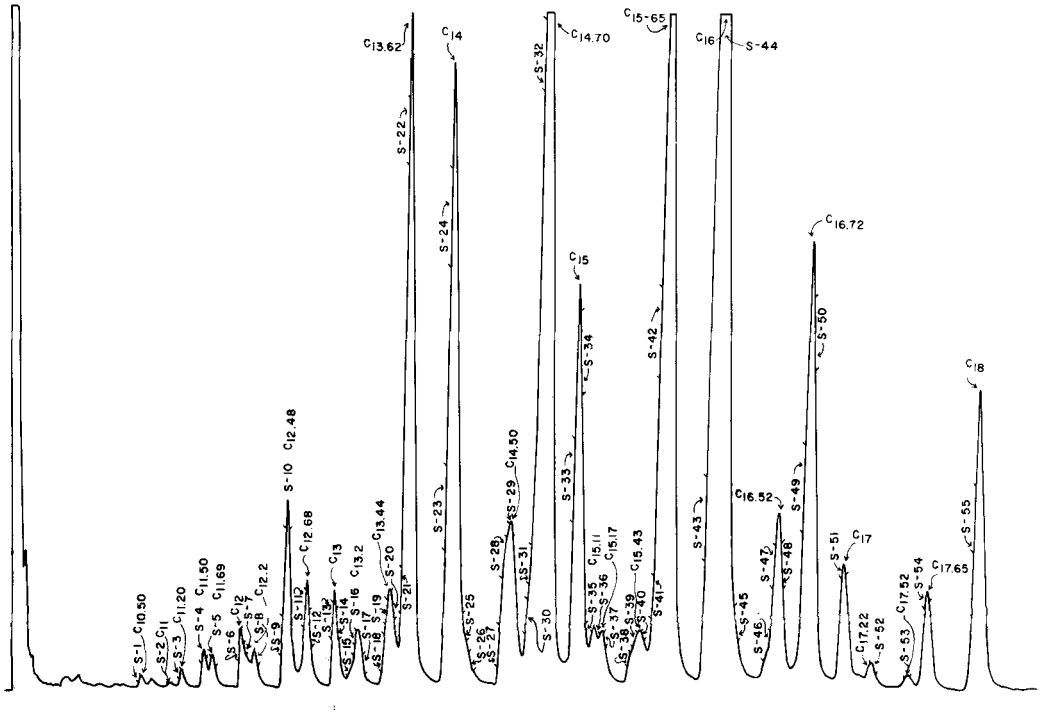

FIG. 1. Gas chromatographic tracing of the saturated fatty acid methyl esters of the wax esters of vernix caseosa showing the points at which a 5-second mass spectrometric scan was made (notches on peaks). The center of these scans are the equivalent chain length values listed in Tables I and III. Gas liquid chromatography was performed on an Aerograph Model 1400 instrument with a 9 ft x 1/8 in. stainless steel column packed with 3% OV-101 on 100-120 mesh Gas Chrom Q (Applied Sciences, Inc., State College, Pa.). It was programmed from 150-270 C at 2 C/min with He at 40 ml/min flowing. Although esters to C_{28} emerged, only the peaks to C_{18} are shown. One-half the effluent went to a flame ionization detector, the other half, interfacing by means of a jet, to a duPont Model 21-491 mass spectrometer operating at 70 eV ionizing potential.

TABLE I

Monomethyl Branched Saturated Fatty Acid Methyl Esters of Wax Esters of Vernix Caseosa

Structures identified	ECL[a]	Scan No.	Base peak	Molecular ion	Main identifying m/e (% of base peak)
4-Me C_{11}	11.49	4	87	214	87,115(7),M-57(10),99(6),127(3),M-73(9)
6-Me C_{11}					M-76(3)
8-Me C_{11}					171(0.4),139(0.8),121(0.8)
10-Me C_{11} iso					M-65(1.1)
10-Me C_{11} iso	11.64	5	74	214	M-65(2.0)
4-Me C_{12}	12.46	9	87	228	87,115(16),M-57(11),M-73(11)
6-Me C_{12}					M-76(18)
8-Me C_{12}					171(=M-57)(11),139(1.5),,121(0.5)
4-Me C_{12}	12.52	10	87	228	87,115(6),M-57(6)
6-Me C_{12}					M-76(1)
8-Me C_{12}					171(6),139(2),121(0.5)
10-Me C_{12} anteiso	12.69	11	74	228	M-29>M-31,M-29(6),M-29-32(2),M-29-32-18(3)
4-Me C_{13}	13.41	18	74	242	87(57),115(11),M-57(9),M-49(23),M-73(2.1)
6-Me C_{13}					M-76(19)
8-Me C_{13}					171(0.7),139(2),121(2.1)
10-Me C_{13}					199(0.9),167(4.7),149(0.7)
4-Me C_{13}	13.47	19	74	242	87(92),115(9),M-57(9),M-49(1.7),M-73(8.6)
6-Me C_{13}					M-76(9.0)
8-Me C_{13}					171(1.0),139(2.4),121(1.8)
4-Me C_{13}	13.50	20	74	242	87(82),115(6.3),M-57(9.5),M-73(8.8)
6-Me C_{13}					M-76(2.0)
8-Me C_{13}					171(1.4),139(1.6),121(0.8)
12-Me C_{13} iso	13.59	21	74	242	M-65(0.8)
4-Me C_{14}	14.47	28	87	256	87,115(10),M-57(6.7),M-49(0.7),M-73(0.6)
6-Me C_{14}					M-76(6.3)
8-Me C_{14}					171(1.1),139(2.7),121(1.2)
10-Me C_{14}					199(6.7),167(0.6),149(0.6)
4-Me C_{14}	14.51	29	87	256	87,115(5.0),M-57(6.8),M-49(0.4),M-73(5.2)
6-Me C_{14}					M-76(1.0)
8-Me C_{14}					171(0.9),139(0.7),121(0.5)
10-Me C_{14}					199(6.8),167(0.8),149(0.7)
13-Me C_{14} iso	14.59	30	74	256	M-65(0.39)
13-Me C_{14} iso	14.65	31	74	256	M-65(0.59)
12-Me C_{14} anteiso	14.73	32	74	256	M-29>M-32
4-Me C_{15}	15.41	39	74	270	87(54),115(2.5),M-57(6.7),M-73(4.8)
6-Me C_{15}				+284	M-76(5.7)
8-Me C_{15}					171(0.9),139(3.5),121(1.7)
10-Me C_{15}					199(0.17),167(1.3),149(0.7)
12-Me C_{15}					227(1.3),195(1.1),177(0.24)
4-Me C_{15}	15.45	40	87	270	87,115(4.7),M-57(9.1),M-73(7.3)
6-Me C_{15}					M-76(0.9)
8-Me C_{15}					171(1.8),139(0.9),121(1.5)
10-Me C_{15}					199(2.0),167(0.3),149(0.9)
14-Me C_{15} iso	15.58	42	74	270	M-65(0.69)
4-Me C_{16}	16.43	47	74	284	87(77),115(7.7),M-57(9.7),M-49(0.52),M-73(5.2)
6-Me C_{16}					M-76(0.8)
8-Me C_{16}					171(1.3),139(1.3),121(2.5)
10-Me C_{16}					199(8.0),167(1.0),149(1.8)
12-Me C_{16}					227(9.7),195(1.3),177(1.4)
15-Me C_{16} iso					M-65(0.58)
4-Me C_{16}	16.50	48	74	284	87(92),115(6.0),M-57(5.0),M-49(0.13),M-73(2.5)
6-Me C_{16}					M-76(0.8)
8-Me C_{16}					171(1.3),139(1.3),121(2.5)
10-Me C_{16}					199(8.0),167(1.0),149(1.8)
12-Me C_{16}					227(5.0)=M-57,195(3.0),177(2.0)
14-Me C_{16} anteiso	16.70	49	74	284	M-29>M-31,M-29(6.5),M-29-32(2.5),M-29-32-18(3.5)
15-Me C_{16} iso					M-65(0.85)
4-Me C_{17}	17.45	53	74	298	87(84),115(8),M-57(7),M-73(5.8)
8-Me C_{17}					171(2.3),139(1.7),121(0.8)
10-Me C_{17}					199(3.3),167(1.7),149(2.3)
12-Me C_{17}					227(3.3),195(2.3),177(1.7)
16-Me C_{17} iso	17.63	54	74	298	M-65(0.06)

[a]ECL, equivalent chain length. If one considers the increase in retention time in going from one normal methyl ester to the next higher homolog as 1.00, then that fraction of this increase in going from the lower normal methyl ester to the center of the 5-second scan is the fractional portion of the ECL values listed. The integer portion of the ECL value represents the number of C-atoms in the fatty acid moiety of the lower normal methyl ester (6).

lipids to avoid contamination problems encountered with human skin surface lipid, especially from bacteria that are known to produce branched acids. We examined the wax esters because they are produced by sebaceous glands only and they are not affected by esterase activity as are other esters (2).

A sample of vernix caseosa (11.82 g) from a Caucasian male yielded 1.370 g of lipid by chloroform-methanol extraction (1). The lipid was chromatographed on the silicic acid column described in (3). After saturated hydrocarbons (trace) were eluted with 505 ml hexane, and squalene with 350 ml 8% benzene in hexane, the sterol ester + wax ester fraction (560 mg) was eluted with 2310 ml 8% benzene in hexane + 450 ml 15% benzene in hexane + 150 ml 20% benzene in hexane. Aliquots of this mixture were separated into wax ester and sterol ester fractions on columns of MgO as described in (4). For 50.8 mg and 56.4 mg aliquots, we recovered respectively 15.8 mg and 19.4 mg wax ester, 1.25 mg and 0.75 mg of an intermediate fraction, 26.6 mg and 29.9 mg sterol ester, and 5.3 mg and 5.0 mg post sterol ester material. A 31.5 mg aliquot of the combined wax esters was then saponified with 10% KOH in 90% ethanol under an atmosphere of nitrogen for 2 hr then the saponified mixture was acidified with 6N H_2SO_4 and the saponified products were extracted with hexane. The unsaponifiables were separated quantitatively from the fatty acids on a column (10 x 1.6 cm) of 11.5 g Florisil, eluting with 110 ml $CHCl_3$ to recover the unsaponifiables (19.45 mg), and with 100 ml $CHCl_3$-methanol-formic acid 90:5:5 to recover the fatty acids (12.05 mg). Thin layer chromatography of these substances on silica gel-$MgSiO_3$ 9:1 developed with hexane-ether 60:40 showed only one spot each for the unsaponifiables and for the fatty acids. The latter were then esterified with BF_3 in methanol and the methyl esters (10.5 mg) separated into saturates (2.66 mg), monoenes (6.85 mg), dienes (0.7 mg) and polar material (0.2 mg) on a $AgNO_3/SiO_2$ column as described in (5). The saturated fatty acid methyl esters were then gas chromatographed on 3% OV-101 with half the effluent going to the hydrogen flame and the other half into a duPont 21-491 mass spectrometer (see Fig. 1 for details).

RESULTS AND DISCUSSION

Figure 1 shows the GLC tracing of the saturated fatty acids of the wax esters of vernix caseosa and the points on the GLC peaks where the 5-second mass spectrometric scans were taken, i.e., S-1, S-2, and so on. It also gives the equivalent chain lengths (ECL) (6) of the tops of the peaks, many of which are obvious mixtures. Interpretation of spectra was based largely on the work of Ryhage and Stenhagen (7) and of Odham who analyzed the preen gland lipids (sebaceous-type glands) from many birds [see Karlsson and Odham (8) and the references listed there]. Table I lists the structures of the monomethyl branched fatty acids (as methyl esters), the main identifying ions on which these structures are based and the ECL of the center of the scan. Although the amounts of each isomer in the mixtures could not readily be determined from the mass spectra, the very large peaks at an m/e on 87 clearly show that, apart from the iso and anteiso derivatives, methyl at position 4 predominates. GLC retention data (Table II) also shed some light in this direction. Note that the 4 methyl isomer has a higher ECL (18.51) than any of the other isomers where the position of the methyl branch is closer to the carboxyl group than the 14 methyl isomer. (The same observation was made by Gordon L. Long in his Ph.D. thesis [with James Cason] who measured the retention times for the 2,3,4,5,7,9,11,13,15 and 17 methyl isomers on high vacuum silicone grease at column temperatures of 237 and 241 C [private communication–James Cason].) Figure 1 shows more material at ECL's of 0.50

TABLE II

Equivalent Chain Length Values of Various Mono Methyl Branched Methyl Octadecanoates on OV-101[a]

Position of methyl branch on C_{18} chain[b]	ECL[c]
2	18.33
3	18.39
4	18.51
6	18.42
8	18.40
9	18.41
12	18.44
14	18.52
15	18.58
16 (anteiso)	18.73
17 (iso)	18.63

[a]Gas liquid chromatography conditions: Beckman GC-4 instrument, 6 ft x 1/4 in. stainless steel column packed with 3% OV-101 on Gas Chrom Q 100-120 mesh with He flowing at 40 ml/min, isothermal at 200 C. Standards injected before and after the test substances showed the same retention to ± 0.02 min.

[b]The 2 through 15 methyl isomers (synthetic) were gifts of J. Cason; the 16 methyl isomer was purchased from Analabs, Inc., New Haven, Conn. The value for the 17 methyl isomer was obtained from a plot of synthetic iso acids (C_{14}, C_{16}, C_{18}, C_{20}) purchased from Analabs, Inc.

[c]See (6) and fn. a, Table I. In this case the fractional increase in retention time to the tops of the peaks was used as is normally done.

TABLE III

Dimethyl Branched Saturated Fatty Acid Methyl Esters of Wax Esters of Vernix Caseosa

Me position	ECL[a]	Scan no.	Base peak	Molecular ion	Main identifying m/e (% of base peak)
4	10.5	1	87	200	87,115(13),M-57(15),M-49(6),M-73(11)
6					M-76(5)
8					M-65(2)
4	11.2	3	87	214	87,115(0.7),M-57(20),M-73(14)
8					M-29)M-31,M-29(2.6),M-29-32(6.2),M-29-32-18(2.6)
4	12.2	8	87	228	87,115(6.5),M-57(10.4),M-73(7.0)
6					M-76(0.5)
8					171(=M-57)(10),139(1.3),121(0.6)
10					M-65(2)
4	13.11	15	87	242	87,115(13),M-57(18),M-73(6.9)
6					M-76(12)
4	13.16	16	87	242	87,115(9.6),M-57(14),M-73(8.3)
6					M-76(1.9)
8					171(0.6),139(2.2),121(1.3)
10					M-29)M-32,M-29(2.9),M-29-32(2.4),M-29-32-18(1.9)
4	13.25	17	87	242	87,115(4.2),M-57(11),M-73(8.3),M-49(2.5)
8					171(2.5),139(2.5),121 absent
4	14.09	25	74	242	87(45),115(6.2),M-57=199(5),M-73(2),M-49(1.2)
6				+256	M-76(16)
8					171(0.8),139(1.3),121(0.8)
10					199(5.0),167(1.0),149(0.03)
12					M-65(0.18)
4	14.14	26	74	256	87(91),115(8.8),M-57(9.4),M-73(6.5)
6					M-76(2.4)
12					M-65(0.65)
4	14.35	27	74	256	87(25),115(7.6),M-57(1.8),M-73(1.5),M-49(1.4)
6					M-76(16.4)
8					171(1.2),139(3.2),121(0.94)
10					199(1.8),167(0.2),149(0.4)
4	15.10	35	74	256	87(25),115(7),M-57(1.8),M-73(1.3),M-49(0.65)
6				+270	M-76(10)
8					171(0.4),139(3.1),121(0.9)
12					227(0.65),195(2.2),171(0.3)
4	15.14	36	74	270	87(63),115(10),M-57(9),M-73(5)
6					M-76(11)
8					171(1.9),139(1.4),121(1.1)
10					199(2.3),167(0.6),149(0.9)
12					227(0.7),195(2.3),177(0.6)
4	15.19	37	74	270	87(88),115(5.2),M-57(15),M-73(10),M-49(0.4)
6					M-76(1.8)
8					171(0.5),139(0.7),121(0.2)
10					199(0.7),167(0.6),149(0.6)
4	16.15	45	74	270	87(80),115(7),M-57(5),M-43(.5),M-73(1.2)
6				+284	M-76
8					171(3),139(.8),121(3)
10					199(5),167(0.3),149(3)
14					M-65

[a]See *fn.* a, Table I.

than at 0.40.

Table III gives the position of the methyl groups in mixtures of the dimethyl isomers. These substances show the same types of spectra as the monomethyl derivatives but their GLC retention data suggest two methyl branches (see [8] for similar examples). Although any two of the methyl groups listed could be present in any molecule, the large amount of methyl at position 4 suggests that this is one of the two positions for most molecules. If we assume that material in the 0.40-0.50 region of ECL values are the monomethyl branched isomers and material in the 0.10-0.20 region of ECL values are the dimethyl branched isomers, then the monomethyl isomers make up 7.6% of the saturates or 1.9% of the total acids and the dimethyl

isomers make up 0.54% of the saturates or 0.14% of the total fatty acids of vernix caseosa wax esters. The branched material listed as dimethyl isomers at an ECL of 14.35 (Table III) appears to be an exception to this classification of mono and dimethyl derivatives.

Human sebum, which makes up a major part of vernix caseosa, differs markedly in lipid composition from the preen gland of most birds (9), However similar mechanisms for the synthesis of the branched chain acids may be involved because the methyl branches of the fatty acids found in this study are, as in preen gland lipids, mainly on the even-numbered carbon atoms. In preen glands the methyl branch is primarily at the 2 position (8), whereas in vernix caseosa it is mainly at the 4 position, indicating an important difference in synthesis. Methyl group appearing at the 2 position in vernix caseosa fatty chains is very low in amount if present at all.

The biosynthesis of methyl branched acids may occur by replacement of malonyl CoA with methyl malonyl CoA at the point of the branch (10,11). Propionyl CoA could in turn be a precursor of methyl malonyl CoA. It could also serve as the initial primer for biosynthesis of the odd straight chain acids (12), another type of acid found in appreciable amount in sebum. Biosynthesis of the anteiso acids could also occur, at least in part, by condensation of acetyl CoA with methyl malonyl CoA, because this would also put a methyl branch on an even C-atom. The presence of iso fatty acids with an *odd* number of C-atoms as in methyl 13-methyltetradecanoate is an exception to this scheme. It suggests that either CH_3-CH(CH_3)-CH_2-CH_2-CoA serves as a precursor, or that decraboxylation of an even-numbered iso acid occurs (methyl 14-methylpentadecanoate in this case).

Hitherto it has been assumed solely on the basis of GLC retention data that acids of the iso and anteiso type occur in human sebaceous material. The presence of these acids in these materials is now established by mass spectral data.

Traces of material with the same ECL values appear in the hydrogenated monoenes of the wax esters and in the saturated fatty acids up to C_{26} of the sterol esters of vernix caseosa (to be reported). They also occur in the glycerides of vernix caseosa and in all the main esters of adult human skin surface lipid. Their occurrence in vernix caseosa establishes them as bona fide human products.

ACKNOWLEDGMENTS

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Field Ionization Mass Spectrometry of Long Chain Fatty Methyl Esters[1]

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ABSTRACT

Mass spectrometry is particularly useful for identifying lipid materials. One primary factor in the interpretation of mass spectra is the recording of the molecular ion peak giving the molecular weight of the compound. Regrettably many compounds, including hydroxy compounds, do not give significant molecular ion peaks; consequently their identification is difficult. A mass spectrometer equipped with a field ionization source produces a greatly different mass spectrum consisting almost entirely of the molecular ion peak. This new source was used to measure the mass spectra of methyl esters of saturated, unsaturated and hydroxy fatty acids. Saturated esters gave the molecular ion peak almost exclusively; unsaturated esters yielded molecular plus metastable ion peaks; whereas the hydroxy esters had molecular, M - 18, metastable and fragment ion peaks.

INTRODUCTION

Mass spectrometry has emerged as an important instrument in the identification of lipid materials. Methyl esters of most fatty acids give excellent spectra including satisfactory molecular ion peaks and many characteristic fragment ion peaks. The mass spectrum of a compound usually gives the molecular weight of the compound from the molecular ion peak and frequently the positions where chains branch and the locations of functional groups. All these features provide a good picture of the structure of the molecule being studied. Regrettably some compounds, such as hydroxy compounds, do not give molecular ion peaks. This limitation decreases the certainty with which a mass spectroscopist can determine the structure of an unknown because he cannot be sure of the over-all size of the molecule. Alternatively, long chain hydroxy acids can be determined by electron impact spectra of the trimethylsilyl derivatives (1) if sufficient sample is available.

At present the electron impact source is used for almost all organic mass spectrometry. This source produces ions by passing the sample vapor through an electron beam with electron energies normally of 70 ev. When an electron hits a sample molecule, it "knocks" an electron out of the molecule and leaves a positive ion with some portion of the 70 ev energy as vibrational energy in the molecule. Since bond energies are only a few electron volts, the collision energy causes considerable fragmentation of the molecule and seldom are the molecular ions among the largest peaks in the spectrum.

The field ion source developed by Beckey et al. (2), Robertson and Viney (3) and others consists of a fine wire or sharp-edged electrode within 0.010 in. of a slit. With about 10,000 v between the edge and the slit, electric fields of the order of 100 million v/cm are generated. This electric field is sufficient to remove electrons from sample molecules adjacent to the edge by a quantum mechanical tunneling mechanism, which adds little vibrational energy to the molecule. Because of the low vibrational energy, positive ions formed by the field ion source do not fragment extensively, and the molecular ion peak is usually the largest peak in the spectrum with fragment ions limited in number and intensity.

Beckey and coworkers have published a series of papers (4-7) giving field ion mass spectra of relatively low molecular weight materials and one paper on natural products (8). Wanless and Glock (9) studied hydrocarbon spectra fairly completely. Schulze et al. (10) have published a field ion spectrum of a mixture of free fatty acids ranging from C_{25} to C_{32}. The purpose of the present work was to determine the nature of field ion spectra of high boiling materials of interest to the lipid chemist.

EXPERIMENTAL PROCEDURE

A field ion source, designed and built by Nuclide Corp., was used in a Nuclide 12-90 G mass spectrometer. The source was similar in construction to that used by Robertson and Viney (3). A standard double-edged stainless steel razor blade was mounted 0.008 in. from a 0.025-in.-wide slit. A glass tube carried the

[1]Presented at the AOCS Meeting, Chicago, October 1967.

[2]No. Mktg. and Nutr. Res. Div., ARS, USDA.

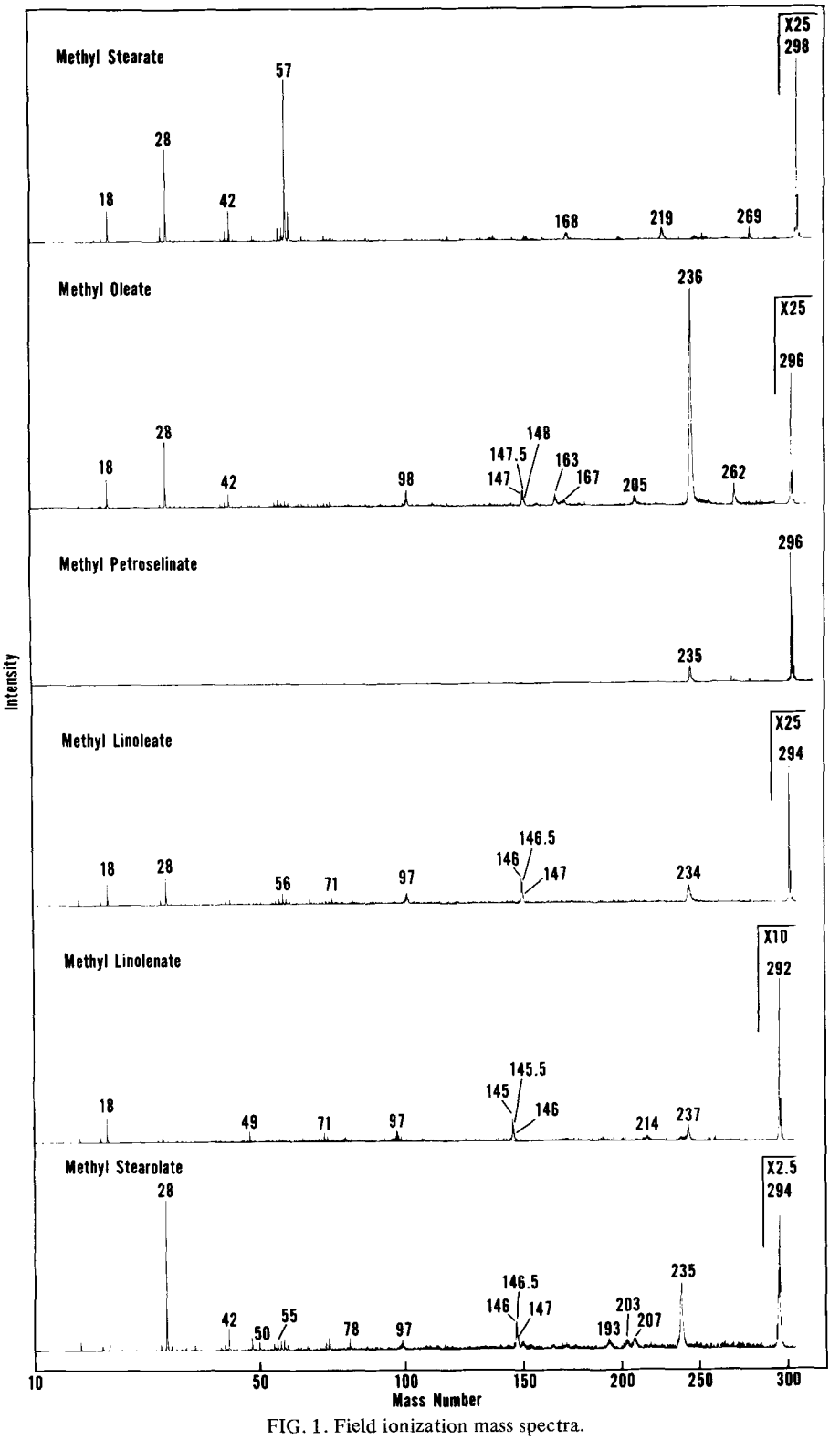
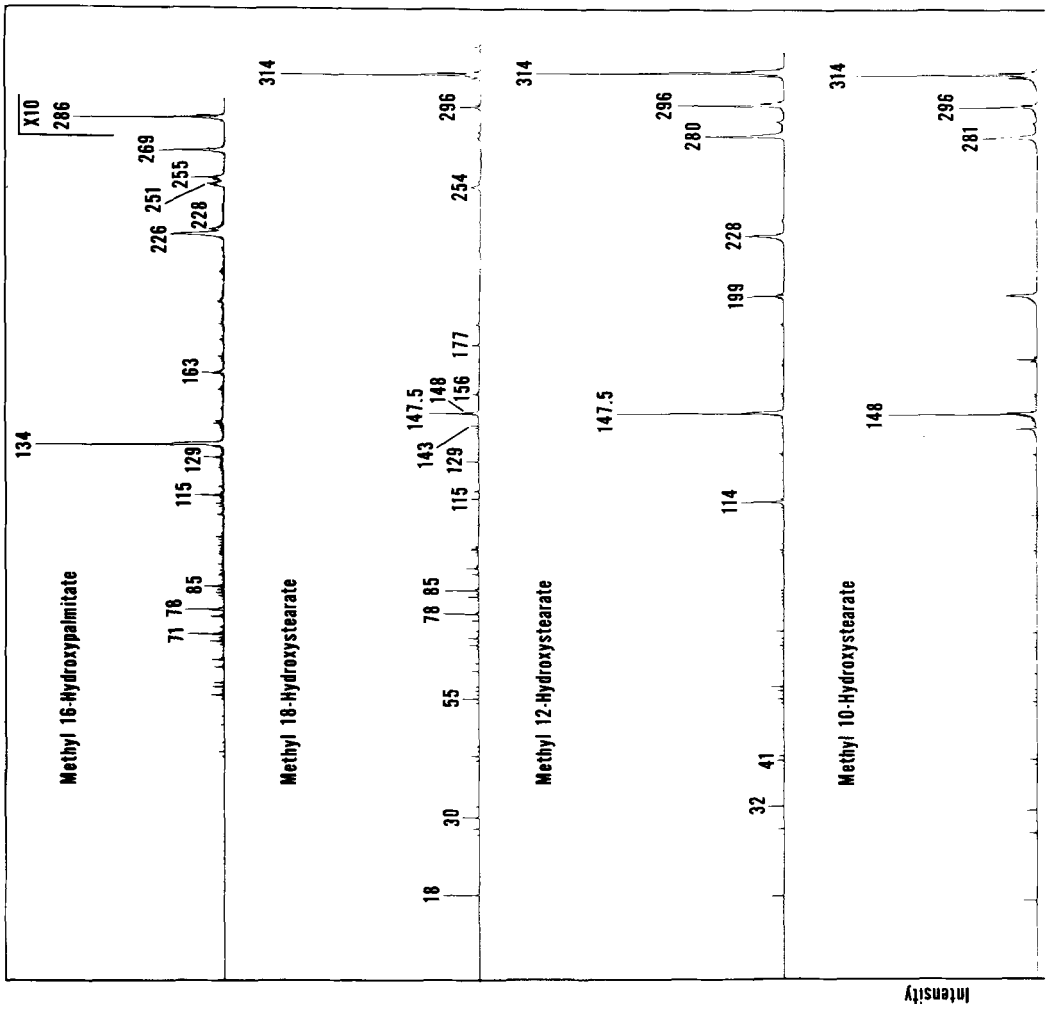


FIG. 1. Field ionization mass spectra.


Methyl 16-Hydroxypalmitate
X10
286
269
255
251
228
226
163
134
129
115
85
78
71
Methyl 18-Hydroxystearate
314
296
254
177
156
148
147.5
143
129
115
85
78
55
30
18
Methyl 12-Hydroxystearate
314
296
280
228
199
147.5
114
41
32
Methyl 10-Hydroxystearate
314
296
281
148
Intensity

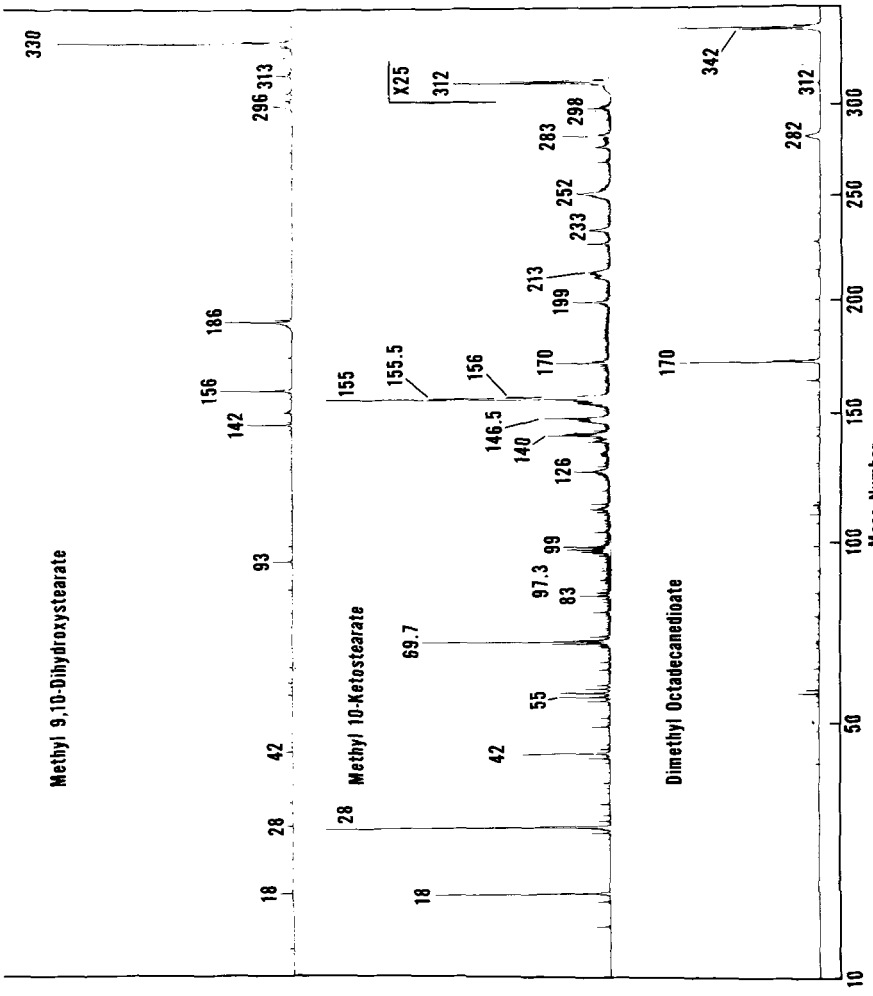


FIG. 2. Field ionization mass spectra.

sample from the inlet and directed it toward the edge of the blade. The inlet and gold foil leak with two 40μ holes were those used with the electron impact source. A spectrum could be obtained with 4-12 kv between the blade and the slit. Field ionization voltages from 6-8 kv produced the best sensitivity with only about 5% variation in intensity over several minutes. A voltage as low as 4 kv gave a spectrum, but sensitivity was low and resolution was poor. In the 9-11 kv region, intensity varied over 40% in short periods and the M - 1 peak was higher than at lower voltages. Considerable arcing took place at 12 kv. The mass spectrum showed a 5% valley at mass 300, with the resolution limited by the source characteristics rather than by the slit widths used.

Mass marking is very difficult and counting is impossible because there are so few peaks. Mass marking was done by carefully comparing the spectrum of a pure compound with the spectrum of a mixture of methyl esters containing molecular ion and isotope peaks at every 14 mass numbers. On the original charts one can see double charged and some triply charged ions, which can be identified because two or three peaks fall equally spaced within one mass unit. In some spectra where singly charged peaks were adjacent to the multiply charged peaks, the latter could be identified to within one-third of a mass unit, but where they are isolated they can usually only be identified within one mass number.

Except for hydroxy, keto and dibasic esters, compounds were purchased commercially and their purity was checked by gas chromatography.

RESULTS AND DISCUSSION

In the 13 spectra shown in Figures 1 and 2, the molecular ion peak is always the largest one seen in the spectrum except in that for methyl octadecane-1,18-dioate. Often the molecular ion peak is 25 times the next highest peak. This very high relative intensity of the molecular ion is the basis for the technique's usefulness in determining molecular weights of unknowns. With the exception of methyl 10-ketostearate, none of the compounds in Figure 2 has a usable molecular ion peak in the electron impact spectrum. Thus methyl 16-hydroxystearate and methyl 18-hydroxystearate give electron impact spectra similar to that of the unsaturated methyl ester without any evidence of either the alcohol group or the true molecular weight. Although electron impact spectra for hydroxy stearates with the hydroxyl in the middle of the chain indicate position of the OH group by cleavage of the carbon chain adjacent to the OH group, the spectra do not indicate chain length because there is no molecular ion peak.

While the molecular ions of field ion spectra are very intense, the presence of M - 1 and M + 1 peaks causes some complications and makes field ion spectra useless for accurate deuterium isotope studies. On the one hand the M - 1 peak is usually small enough not to be troublesome in qualitative work, but on the other hand the M + 1 peak can be as large as or larger than the molecular ion peak. This situation is seen in the spectrum of methyl octadecane-1,18-dioate where the M + 1 peak is larger than the M peak. Intensity of this M + 1 peak can be varied over a wide range by varying the edge-to-slit voltage. Higher voltages tend to increase the M + 1 peak.

Since background was not detectable with this field ion source, peaks at 18 and 28 come from the samples. Field ion spectra show several metastable ions for each compound—i.e., ions that fragment after leaving the source but before entering the magnetic field. Almost all of the metastable ions correspond to logical fragmentations if the parent ion of the fragmentation is assumed to be the molecular ion.

The molecular ion peak of methyl stearate (Fig. 1) is 25 times as intense as the next highest peak in the spectrum. Respective intensities of the field ion peaks M - 1, 5.6%, and M + 1, 24.5%, compare with 0.75% and 21.18% in the electron impact spectra. The methyl stearate spectrum has simple fragment peaks at masses 18, 28, 42 and 57. Metastable peaks occur at 269, caused by loss of CH_3; 219, loss of 42 mass units; and 168, loss of 74 mass units.

Methyl oleate has a molecular ion region comparable to the methyl stearate peaks, but it also has a large metastable peak at 236 due to a loss of 32 mass units, probably CH_3OH. Peaks at 147, 147.5 and 148, half a mass unit apart, indicate doubly charged mass fragments of 294, 295 and 296 respectively. Although mass marking of these peaks is difficult, wherever the mass marking is most reliable the highest mass of the three doubly charged peaks is one-half of the molecular ion peak. Triply charged peaks are also seen in some spectra. All five of the hydroxy compounds (Fig. 2) have double charged peaks equivalent to (M - 18)/2 or the molecular ion minus water double charged.

The electron impact spectra of the hydroxy compounds all have intense peaks due to cleavage of the carbon chain adjacent to the hydroxyl branch. Peaks corresponding to these simple cleavages can also be seen in the field ionization spectra.

Peak 170, the largest peak in the spectrum

of methyl octadecane-1,18-dioate, is a part of a set of three doubly charged peaks with the least intense peak being 171, which is one-half of the molecular ion 342.

In summary, then, one great weakness of mass spectrometry is that some compounds do not give a molecular ion peak in the electron impact source; this lack causes considerable trouble in the interpretation of all spectra because one can never be absolutely sure that the highest mass number in the spectrum of a pure unknown is the molecular ion peak. The field ion source with its very intense molecular ion peak relieves this problem and allows the molecular weight of a compound to be determined with great confidence.

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Metabolism of Labeled Isomeric Octadecenoates by the Laying Hen[1]

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ABSTRACT

Discrimination between octadecenoic acid isomers by the laying hen has been studied using tritium (^3H), carbon-14 (^{14}C) and deuterium (d) labeled oleate and elaidate esters. Hydrogen isotopes were positioned at the double bond, whereas ^{14}C was located in the 1-, carboxyl carbon. The egg acted as a biological trap, providing an automatic daily biopsy with which to study the metabolism of the fed isomers. Monitoring the incorporation of isomers was facilitated by dual label feeding experiments, and $^3H/^{14}C$, d_2/d_o and d_2/d_1 ratios were determined on the isomeric mixtures fed, on the total egg lipids extracted and on the isolated neutral lipid and phospholipid fractions. Comparison of isotopic ratios of the fed mixture and of the lipid fractions provided an evaluation of discrimination by the hen during the transport of isomeric octadecenoates into the egg lipids. Radioactive and stable isotope ratios determined for the neutral lipid indicated a preferential incorporation of the *cis* isomer. Stable isotope ratios determined for the phospholipid showed that the *trans* isomer is preferentially incorporated. The $^3H/^{14}C$ ratios for the phospholipid recovered in each experiment increased greatly whichever isomer was labeled with ^3H, indicating an elimination of the 1-^{14}C-label. Gas liquid radiochromatographic separation of the methyl esters from the neutral lipids and phospholipids showed that the isotopic labels were present almost exclusively in the octadecenoic acid constituent.

INTRODUCTION

Geometric as well as positional isomers of unsaturated fatty acids are formed during the catalytic hydrogenation of vegetable oils to produce commercial oils and shortenings (1). Investigations of the metabolism of *trans* monoenoic acids, principally elaidic acid, have given conflicting results. Transfer of *trans* fatty acids across placental membranes, amniotic and allantoic, has been reported by McConnel and Sinclair (2) with rats, Ono and Fredrickson (3) with rats and dogs, Kaufmann and Mankel (4) with hens, Billek (5) with rats and dogs and Le Breton and Le Marchal (6) with rats and hens. Johnston, Johnson and Kummerow (7) with rats and Sgoutas and Kummerow (8) with humans have reported the nontransfer of *trans* fatty acids.

In all the cited investigations that report transfer across the membranes, the rate of incorporation of elaidic acid was of the same order of magnitude as for oleic acid. Although most of the investigators cited above did not compare the incorporation of *trans* fatty acids into the triglyceride and phosphatide fractions of the lipid, Kaufmann and Mankel (4) reported that the *trans* unsaturated fatty acid content of the triglyceride was always greater than that in the corresponding phosphatides.

In investigations considering metabolic uses of *trans* fatty acids other than transfer across placental membranes, Coots (9) reported that elaidic acid showed a slightly greater incorporation into the lymph phospholipid fraction than did oleic acid. Sinclair (10,11) reported a similar tendency for elaidic acid to be incorporated into the phospholipid fraction of blood and other tissues.

In the present investigation, mixtures of isotopically labeled oleic and elaidic acid esters were fed to a laying hen. Each geometric isomer was labeled with tritium (^3H), radiocarbon (^{14}C) or deuterium (d). The labeled isomers were fed in pairs or groups, with each individual labeled differently, to permit measurements of ratios of $^3H/^{14}C$ and ratios of d_2/d_o or d_2/d_1, or both. The egg served as a "biological trap" yielding an automatic daily biopsy with which to study the metabolism of the isomer in the hen. Relative rates of transfer of isomers across membranes and their incorporation into lipids were indicated by the isotopic ratios determined for the fed mixture, the total egg lipid, the neutral lipid and the phospholipids. The dual label design improves the precision and the accuracy of experiments because exogenous fatty acid metabolism is not confused with the endogenous.

[1]Presented at the AOCS Meeting, Houston, May 1971.

[2]No. Mktg. and Nutr. Res. Div., ARS, USDA.

EXPERIMENTAL PROCEDURES

Materials

Oleic acid-1-^{14}C was purchased from Amersham-Searle Co., Des Plaines, Ill. Specific activity was 57.8 mc/mM, <1.0% *trans*. Oleic acid was esterified using diazomethane. A single peak was indicated by a radiochromatographic scan of thin layer chromatographic (TLC) plate.

Tritiated water (HTO) was purchased from Amersham-Searle Co. Specific activity was 3.6 C/mM.

Oleic acid: The urea-half soap crystallization procedure (12) was used to prepare 110 g of oleic acid from 780 g of olive oil. The purified oleic acid contained less than 0.5% palmitic acid and less than 0.5% linoleic acid impurities.

Stearolic acid: Oleic acid (20 G) was brominated and dehydrohalogenated at 200 C using KOH in ethylene glycol. The reaction mixture was diluted with H_2O, acidified and extracted twice with petroleum ether (PE). Crystallization of the PE extract from ethanol-water at 4 C yielded 10.5 g of pure stearolic acid.

Methyl oleate 9,10-^{3}H: Stearolic acid was esterified with diazomethane. One hundred milligrams of copper chromite catalyst (Harshaw Chemical Co.) was stirred with 16 μl of HTO and H_2 gas at atmospheric pressure for 1 hr at 195 C. Approximately 1.0 g of methyl stearolate was injected into the reaction vessel through a rubber septum. Stirring was continued for 2 hr while H_2 was maintained at atmospheric pressure. The product was filtered through a medium sintered disk funnel. Gas liquid radiochromatography (GLRC) (13) gave mass and radioactivity tracings which coincided and which were the same as a methyl oleate standard. Ozonolysis-pyrolysis (14) of the product yielded radioactive fragments identified by GLRC as 9-aldehyde and 9-aldehyde ester. IR analysis indicated that the product contained less than 1% *trans* isomer. Such stereospecific hydrogenation of stearolate with copper catalysts was reported by Koritala (15). Approximately 95% of the tritium was located at the double bond position as determined by bromination, dehydrohalogenation and assay of the hydrogenation product.

Radioactivity of the product was assayed by dissolving weighed samples in 10 ml of toluene scintillation solvent [4 g of 2,5-diphenyl oxazole (PPO) per liter] using a Beckman LS-250 scintillation counter. The specific activity was determined to be 5.27 mc/mM.

Ethyl oleate-9(10)-d_1: Ethyl oleate-d_1 was prepared from methyl stearolate according to the following procedure (16). A 250 ml, three-necked, round-bottomed flask was equipped with thermometer, dropping funnel and drying tube. The glassware was thoroughly flame-dried before being used. Sodium borohydride (2.05 g) was mixed with 40 ml of diglyme freshly distilled from $LiAlH_4$ and cooled to ca. 3 C. Next 15.2 ml of 2-methyl-2-butene was mixed with 25 ml of dry diglyme and added to the sodium borohydride. After cooling to ca. 0 C, 9.2 ml of boron trifluoride-etherate (72 mmole) in 20 ml of dry diglyme was added over a period of 20 min while holding the reaction temperature at ca. 10 C. The mixture was then stirred at 3 C for 3 hr. Methyl stearolate (15.2 g) dissolved in 20 ml of diglyme was added during a 10 min period, stirred at 0 C for 1 hr and stirred at room temperature for 3 hr. Next the solution was cooled to 3 C and 15 ml of deuteroacetic acid was added at a rate of 1 ml per min. The solution was stirred at 3 C for 3 hr and the mixture diluted with 600 ml of ice water and extracted three times with petroleum ether (PE). The PE extract was washed with $NaHCO_3$, then H_2O and dried over Na_2SO_4 and the solvent evaporated. After standing several days at -25 C, the methyl oleate-9(10)-d_1 became cloudy and a small amount of white precipitate was filtered out. The sample was transesterified using freshly prepared sodium ethoxide and distilled at 138 C under 0.1 mm pressure of Hg. Ethyl

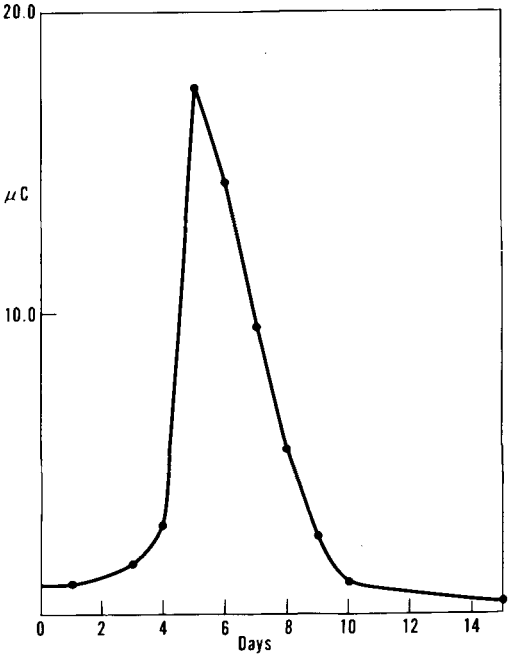

FIG. 1. Incorporation of ^{3}H- and ^{14}C-labeled fatty esters into egg lipid.

TABLE I

Labeled Ester Mixtures Fed to Hen

Feeding	Labeled esters	Total μc		Isotopic ratios	
		^3H	^{14}C	$^3H/^{14}C$	d_2/d_1 or d_0/d_2
1	[a]Oleate-9,10-^3H + oleate-1-^{14}C	924.3	482.0	1.93	
2	[a]Elaidate-9,10-^3H + oleate-1-^{14}C	830.0	113.0	7.31	
3	[a]Elaidate-9,10-^3H + oleate-1-^{14}C [b]+ Elaidate-9,10-d_2 + Oleate-9,(10)-d_1	375.0	500.0	0.75	1.00
4	[a]Oleate-9,10-^3H + elaidate-1-^{14}C [b]+ Elaidate-d_0 + oleate-9,10-d_2	521.2	176.8	2.95	1.00

[a]Methyl esters.
[b]Ethyl esters.

oleate-9(10)-d_1 was 99+% pure as analyzed by GLC and TLC.

Ethyl oleate-9,10,d_2: Stearolic acid (4 g) was esterified using absolute ethanol with H_2SO_4 catalyst. The ethyl stearolate was reduced to ethyl oleate-9,10-d_2 using deuterium–Lindlar catalyst (17,18). The procedure consisted of mixing 0.4 g of Lindlar catalyst, 55 ml of PE and 1.0 ml of quinoline. The catalyst was subsequently reduced with deuterium gas for 30 min or until deuterium uptake stopped and the solution was filtered through celite. Quinoline was washed off with dilute HCl, and the ethyl oleate-9,10-d_2 remained in solution. GLC and TLC analysis showed the product contained less than 0.3% stearate and stearolate. Ninety per cent of the ethyl oleate-9,10-d_2 contained two dueteriums per molecule-9,10-d_2 contained two deuteriums per molecule as analyzed by mass spectroscopy (MS).

The hydrogenation procedure was repeated using hydrogen gas and methyl stearolate. IR analysis of the reduction product (methyl oleate-d_0) detected no *trans* isomers.

Elaidic acid esters: Oleic acid (14.3 g) or ethyl oleate-9,10-d_2 (7 g) was elaidinized using nitrous oxide (19). The elaidinized oleic acid was then fractionally crystallized from 100 ml of acetone at -27 C (20). Crystallization yielded 6.2 g of 99% elaidic acid which was esterified using absolute ethanol with sulfuric acid catalyst. Two-gram portions of the elaidinized ethyl oleate-d_2 were separated into ethyl elaidate-d_2 and ethyl oleate-d_2 fractions by silver-ion exchange resin chromatography (21). Analysis by mass spectrometry confirmed that no hydrogen-deuterium exchange occurred during the nitrous oxide isomerization.

Methyl oleate-1-^{14}C or methyl oleate-9,10-^3H was eladinized by a micromodification of the nitrous oxide technique (19). To a micro flask fitted with a rubber septum and magnetic stirring bar was added 10–50 mg of labeled methyl oleate. The temperature was raised to 65 C, and 1–5 μl of 2M $NaNO_2$ was injected into the falsk followed by 1–4 μl of 6M HNO_3; the mixture was stirred vigorously for 10 min. The products were dissolved in PE and washed with water. The solution was passed through a short silicic acid column to remove nitrogenous byproducts and to recover the isomerized fatty ester. The labeled *trans* monoene was separated

TABLE II

Radiochemical Analysis, Egg Neutral Lipids

Experiment	Fed esters[a]	Specific activity μc/mg X 10^{-3}		Isotopic ratios ($^3H/^{14}C$)	
		^3H	^{14}C	Fed	Recovered neutral lipid
1	Oleate-9,10-^3H + oleate-1-^{14}C	8.80	4.64	1.93	1.96
2	Elaidate-9,10-^3H + oleate-1-^{14}C	5.93	0.95	7.31	6.22
3	Elaidate-9,10-^3H + oleate-1-^{14}C	2.40	5.07	0.75	0.62
4	Oleate-9,10-^3H + elaidate-1-^{14}C	3.24	0.61	2.95	5.36

[a]Methyl esters.

TABLE III

Radiochemical Analysis, Egg Phospholipids

Experiment	Fed esters[a]	Specific activity μc/mg X 10^{-3}		Isotopic ratios ($^3H/^{14}C$)	
		^3H	^{14}C	Fed	Recovered phospholipid
1	Oleate-9,10-^3H + oleate-1-^{14}C	38.01	1.38	1.93	27.59
2	Elaidate-9,10-^3H + oleate-1-^{14}C	17.88	1.08	7.31	16.55
3	Elaidate-9,10-^3H + oleate-1-^{14}C	4.87	4.99	0.75	0.88
4	Oleate-9,10-^3H + elaidate-1-^{14}C	5.29	0.32	2.95	16.59

[a]Methyl esters.

from the residual *cis* monoene by silver-ion exchange resin chromatography (21). The effluent was monitored by a flow-through cell accessory to the Beckman LS 250. The cell was packed with europium-activated calcium fluoride as the scintillator. Recovery of *trans* monoene was approximately 60%.

Animal Experiment

In the absence of elaborate metabolism chambers, an adequate area for maintenance of a white leghorn hen was devised. A wire cage fitted with a feeding trough and watering device was mounted above a stainless steel tray in a laboratory hood with continuous airflow. The tray was equipped with a drainage tube for easy recovery of radioactive feces or their disposal after monitoring by water flushing. The hen was maintained on a diet of "Critic" laying mash (Citric Mills, Inc., Beardstown, Ill.) throughout the experiments.

Mixtures of isomers were prepared from the ^{14}C and ^3H radioactive esters alone and from the deuterium as well as ^{14}C and ^3H labeled esters as shown in Table I. Approximately 50 mg of the radioactive esters were administered in a 150 μl gelatin capsule placed in the gullet of the hen. Approximately 6 g of the mixed isotopes were force-fed using a 5 ml syring fitted with a 1.5 in. No. 18 hypodermic needle tightly inserted into a 5-in. segment of Teflon tubing. The syring plus fatty esters was tared, and the esters were administered by inserting the tubing approximately 4 in. down the hen's gullet in order to bypass the trachea.

Eggs laid subsequent to the feeding of the labeled isomers were collected. With each egg the yolk was separated from the white, and the egg lipid was extracted by stirring the yolk with a 50:50 mixture of chloroform:methanol for 1 hr. Coagulated protein was allowed to settle, and the lipid solution was decanted. The residue was washed with the solvent and filtered. This filtrate was added to the previously decanted lipid solution, the solvent was evaporated, and the weight of total lipids was determined. Egg lipids were fractionated by silicic acid chromatography (22) using chloroform:benzene (70:30) followed by 100% methanol as eluting solvents. Fractions collected were analyzed by TLC on Silica Gel G with chloroform:benzene (70:30) solvent. Neutral lipid and phospholipid were isolated free of other constituents. These lipid fractions were used to determine the ratio of incorporation of isomers.

Radiochemical analysis. Dual isotope assay was performed using the three-channel liquid scintillation counter. This instrument is designed to give a counting efficiency of 80% for

TABLE IV

Stable Isotope Analysis, Egg Lipids

Experiment	Fed esters[a]	Isomeric ratios (E1/01)		
		Fed	Recovered neutral lipid	Recovered phospholipid
3	Elaidate-9,10-d_2 + oleate-9,(10)-d_1	1.00	0.82	1.17
4	Elaidate-d_0 + oleate-9,10-d_2	1.00	0.56	1.09

[a]Ethyl esters.

^{14}C and 40% for ^{3}H with less than 8% overlap of the ^{14}C-channel into the ^{3}H-channel.

The lipids were transesterified by the sodium methoxide procedure (23) and the methyl esters analyzed by the gas liquid chromatography-liquid scintillation counting (GLC-LSC) procedure described by Thomas and Dutton (24).

Stable isotope analysis. An Aereograph Autoprep (Model No. A-700) was used to isolate ca. 10–15 mg of monoene from each fraction for IR and MS analysis. Baseline separation of components was achieved using a sample size of 12 to 15 μl on a 10 ft X 3/8 in. aluminum column packed with 10% EGSS-X on Chromosorb P, with a column temperature of 180 C, and a helium flow rate of 80 ml/min was used.

IR analysis for elaidate-d_o was accomplished using a Perkin-Elmer 621 grating IR spectrometer. The procedure used was essentially the same as described by Allen (25) except for two differences: a 50 μl, 0.5 cm micro cell was used rather than the normal IR cell, and the ratio of absorbance at 10.4 vs. 9.2 μ was plotted against the per cent *trans* instead of the absorbance ratio 10.5 vs. 8.6 μ. A standard curve was prepared for 1-20% *trans* using known mixtures of oleate and elaidate. Values from three analyses were averaged, but inherent errors by this procedure were ± 0.4% at low *trans* values.

Monoenes separated by preparative GLC were reduced to methyl stearate using anhydrous hydrazine (26) and the per cent monodeutero stearate or dideutero stearate, or both, in these samples was determined by MS. Hydrazine was used to insure that there was no hydrogen-deuterium exchange during reduction. Isolation and reduction of the monoene improves the sensitivity of the mass spectrometric analysis by (1) increasing the per cent deuterium in the sample analyzed by about three times, (2) eliminating interference of traces of stearate-d_o in the determination of oleate-d_2 by analyzing for stearate-d_2 and (3) being more sensitive and reproducible for stearate than for oleate. The deuterated samples were analyzed six times and the average values at masses 299 (MeSt-d_1) and 300 (MeSt-d_2) were calculated. These values were corrected for the contribution of methyl stearate d_o to the mass 299 and 300 peaks due to the natural ^{13}C abundance. Highly purified methyl stearate-d_o was run before and after analysis of the deuterated methyl stearate to obtain values for the mass 299 and 300 peaks.

RESULTS AND DISCUSSION

Liquid scintillation assay of the total lipids extracted from the eggs showed that the radioactive content increased rapidly until the fifth day and then decreased to background by the thirteenth day after force feeding (Fig. 1). MS analysis of egg lipids recovered during stable isotope experiments confirmed this incorporation pattern. As described by Card and Nesheim (27), the hen develops several yolks simultaneously varying in size from ca. 6-40 mm in diameter. During any one 24 hr period the yolk increases about 4 mm in diameter. Thus the labeled isomers are incorporated into lipid of several yolks at the same time. The amount of incorporation is a function of the over-all size of the yolk at the time the labeled material is fed; that is, the larger the yolk the greater is the incorporation of labeled isomers. An average of 3.0 g of egg lipid was recovered from each egg. consisting of about 90% neutral lipid and 10% phospholipid. Approximately 6% of the total radioactivity administered orally was recovered in the egg lipids. The $^{3}H/^{14}C$ ratio of the total lipid was about the same as that for the mixture fed, indicating that the *cis* and *trans* isomers are transferred across membranes at the same rate.

Only low levels of radioactivity were detected in the feces; the remainder of the radioactive material is assumed to be respired or deposited in the depot fat. Coots (9) has reported the respiration of almost 70% of radioactivity within 51 hr of its administration to rats. Approximately 6% of the deuterium fed to the hen was also recovered in the egg lipid.

The radiochemical analysis of the neutral lipid fractions in each experiment is presented in Table II, and the corresponding analysis for the phospholipid fraction is presented in Table III.

In all radioactively labeled acids the tritium was located at the double bond, whereas the carboxyl group carbon was labeled with ^{14}C. Oleate-1-^{14}C and oleate-9,10-^{3}H were fed in Experiment 1 to determine if the position of the label has any effect on the $^{3}H/^{14}C$ ratio of the incorporated fatty ester. The ratio determined for the neutral lipid was the same as that of the sample fed; indicating that during incorporation of oleate into this lipid fraction there is no isotopic discrimination or loss. However the $^{3}H/^{14}C$ ratio determined for the phospholipid is about 14 times as great as that fed, showing that ^{14}C has been lost. The data indicate that incorporation of the fed methyl oleate into phospholipid involves a metabolic reaction that is not involved in the incorporation into the neutral lipid. Therefore previous conclusions drawn from metabolic data, which were based on a comparison of rates of incorpo-

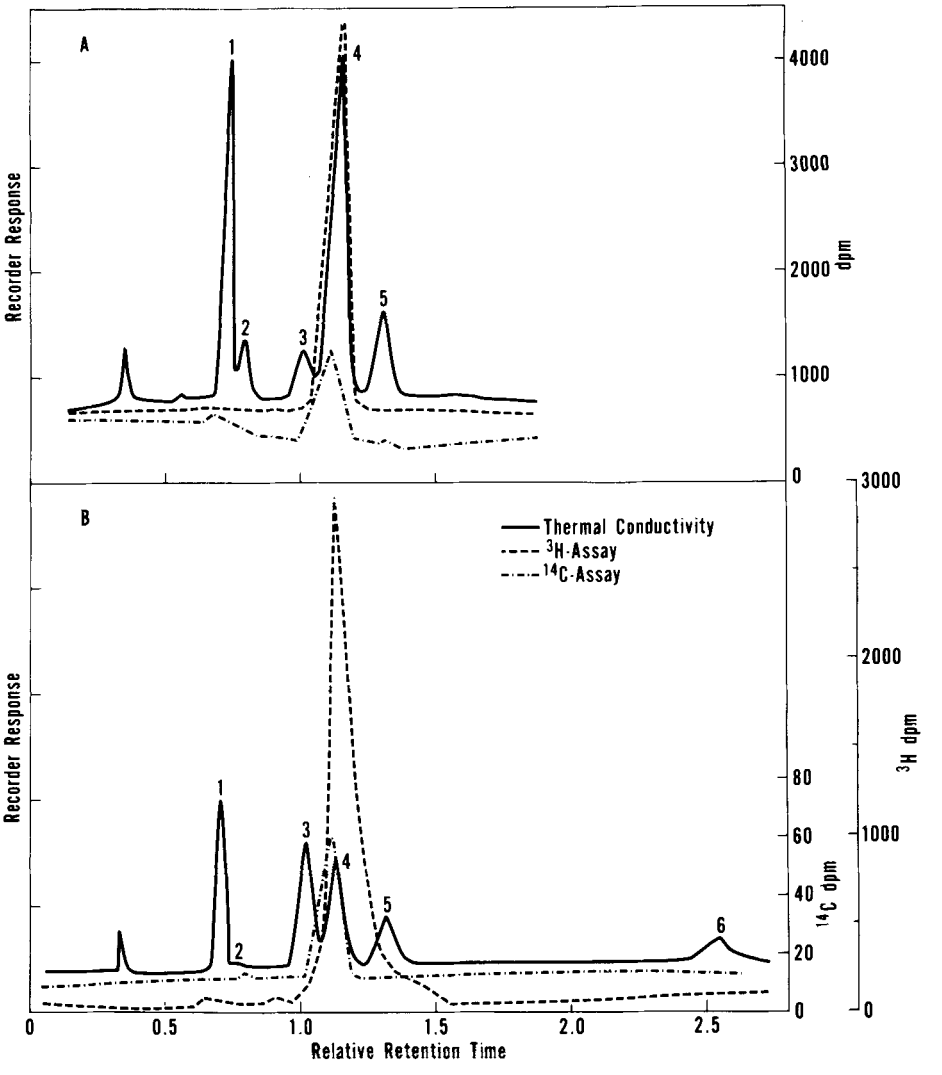

FIG. 2. Gas liquid chromatography-liquid scintillation counting of methyl esters of fatty acids from the (a) neutral lipids and (b) phospholipid fractions. The column was 11% EGSS-X (10 ft X 1/4 in. aluminum) operated at 175 C with a helium flow rate of 30 ml/min. Major peaks are (1) palmitate, (2) palmitoleate, (3) stearate, (4) oleate, (5) linoleate and (6) arachidonate.

ration of 1-^{14}C labeled fatty acids into neutral lipids and phospholipids, should be reevaluated.

When the *trans* isomer labeled with ^{3}H was fed in mixture with ^{14}C-*cis* isomer (Experiments 2 and 3, Table II), the ^{3}H/^{14}C ratio of the neutral lipid decreased, signifiying a partial preferential incorporation of the *cis* isomer. With the labeling reversed—that is ^{3}H-*cis* and ^{14}C-*trans* (Experiment 4, Table II)—the neutral lipid ^{3}H/^{14}C ratio was found to increase, again a preferential incorporation of the *cis* isomer. This preference was also shown by the results of the dual-labeled, stable isotope experiments (Table IV).

Owing to the apparent elimination of ^{14}C during decarboxylation reactions leading to the incorporation of the fed esters into the phospholipid, radiochemical analysis was misleading in determining the ratio of isomers incorporated. Therefore this isomeric ratio was determined by use of a stable isotope. Deuterium located at the 9,10 positions in the carbon chain is not subject to loss due to decarboxylation-recarboxylation. These results, shown in Table IV, indicated that there was a partial, preferential incorporation of the *trans* isomer in the phospholipid.

The tritium specific activity (Table II and

III) of the phospholipid was consistently higher than that of the neutral lipid. This result would be expected since dilution of the incorporated radioactivity with inactive material is much less in the phospholipid which comprises only 10% of the total lipid of the egg.

Curves for the GLC-LSC analysis of the methyl esters prepared from the neutral lipid (curve A) and the phospholipid (curve B) are presented in Figure 2. The plot of the radiochemical data superimposed on the thermal conductivity curve shows that the ^{3}H- and ^{14}C-labels are almost exclusively associated with the octadecenoic acid constituent in each lipid fraction. MS analysis of isolated fatty esters from each lipid fraction showed that the deuterium label was also associated entirely with the octadecenoic acid constituent.

Although incorporation of the fed esters into the neutral lipid appears to involve hydrolysis and direct acylation, the reactions leading to incorporation into the phospholipid appear to be more complex. Double dual-labeled experiments reveal that although the fatty acid molecule is partially degraded (as indicated by the loss of ^{14}C-carboxyl carbon) the degraded molecule can be elongated to C_{18} and incorporated into the phospholipid (as indicated by the appearance of tritium and deuterium in the octadecenoic acid constituent only). This observation is not inconsistent with Lynen's (28) description of the fatty acid cycle an elongation of the partially degraded molecule by condensation with malonyl thioester and subsequent incorporation into the phospholipid.

The present experiments have shown the applicability of the dual-labeled isotope technique for elucidation of metabolic reactions. The hen has been shown to transfer *cis* and *trans* isomers across membranes at about the same rate and to preferentially incorporate the *cis* isomer in the neutral lipid and the *trans* isomer in the phospholipid. Incorporation into the phospholipid involves a reaction that results in the loss of the carboxyl carbon. Deuterium isotope techniques developed for these experiments should find application to human nutrition studies in which radiochemicals cannot be used.

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Alcoholysis, Saponification and the Preparation of Fatty Acid Methyl Esters[1]

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ABSTRACT

Evidence is offered to support the contention that methanolysis precedes the saponification of esters in methanolic solutions of sodium hydroxide. This results from the hydroxide-alkoxide equilibrium which greatly favors methoxide formation even in the presence of rather considerable amounts of water. Saponification-reesterification methods of methyl ester formation are shown to be actually extensions of methanolysis procedures. A simplified method is proposed for the preparation of fatty acid methyl esters.

INTRODUCTION

Saponification and Alcoholysis

The saponification of esters in solution in alcoholic base proceeds slowly in comparison to their alcoholysis. This well-documented feature of ester chemistry is frequently overlooked, although it has important implications with respect to the preparation of fatty acid methyl esters. As early as 1920, Pardee and Reid (1) demonstrated that low saponification numbers can result from a loss, through inefficient condensers, of ethyl esters formed rapidly when glycerides and other esters are dissolved in aqueous 95% ethanolic potassium hydroxide. In 1954, Formo (2) summarized as follows: "During the course of the saponification value determination, using alcoholic alkali, alcoholysis proceeds far more rapidly than saponification with the result that the alkali reacts primarily with ethyl esters rather than triglycerides." Markley (3, p. 864) cites the work and conclusions of Pardee and Reid. However (Ibid. p. 870), he states "It was found [by Kurz (4), Rowe (5) and Toyama and coworkers (6)] that alcoholysis catalyzed by potassium hydroxide consisted of two reactions, namely, saponification followed by reesterification." As will be shown, this view of alcoholysis is incorrect.

The Hydroxide-Alkoxide Equilibrium

Pardee and Reid deduced from their own titration data as well as those of Anderson and Pierce (7) that alcoholysis proceeds faster than saponification. They concluded that the relative difference in rates is of the order of 1500-fold but made no comment concerning the probable reason. It would of course results at least in part from the well-known equilibrium shown in Reaction 1 (below). Even in the presence of very appreciable amounts of water, Reaction 1

Reaction 1: $NaOH + ROH \rightleftarrows RONa + HOH$

proceeds to the right, as written, so that alkoxide predominates. Bender and Glassom (8) found that in alcoholic solutions of sodium hydroxide containing 30% w/w of water, the amount of base present as hydroxide is 52% in ethanol and only 8% in methanol. Caldin and Long (9) had observed that a 0.1 M solution of sodium hydroxide prepared in ethanol containing initially 1% of water consisted of 4.1% hydroxide and 95.9% ethoxide. As the initial water content was decreased to zero the hydroxide content decreased to 0.8% of the total base present. It may be inferred from these studies that essentially all of the dissolved hydroxide is converted to methoxide in methanol solutions containing several per cent of water.

Since alkoxides cause the alcoholysis of esters, Reaction 2, and hydroxide their saponification, Reaction 3, it is not surprising, on the basis of relative concentrations alone, that alcoholysis should be the predominant reaction when an ester is dissolved in a solution of sodium or potassium hydroxide in methanol. Further, the work of Bender and Glassom (8)

Reaction 2: $R'\overset{\overset{O}{\|}}{C}OR'' + Na^+ OR^- \rightleftarrows \left[R'C(=O)(OR)(OR'')\right]^- Na^+ \rightleftarrows R'\overset{\overset{O}{\|}}{C}OR + Na^+ OR''^-$

Reaction 3: $R'\overset{\overset{O}{\|}}{C}OR'' + Na^+ OH^- \rightleftarrows \left[R'C(=O)(OH)(OR'')\right]^- Na^+ \rightarrow R'\overset{\overset{O}{\|}}{C}O^+ Na^- + HOR''$

[1]Scientific Journal Series 7706, Agricultural Experiment Station, University of Minnesota, St. Paul, Minnesota 55101.

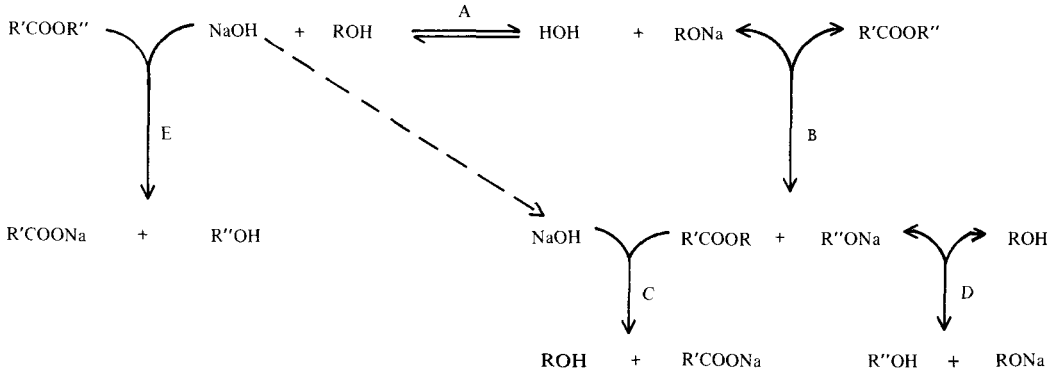

FIG. 1. The alcoholysis and saponification of an ester in an alcoholic base.

indicated that the rates of attack on an ester by hydroxide, methoxide and ethoxide are in the ratios of 1:1.5 9:4.17 respectively. Although these values were obtained by the extrapolation of their data to pure water and may have little relevance when applied to a consideration of the reactions in almost pure alcohol they do indicate that the rate constants are of the same order of magnitude so that the major factors to be considered are indeed the relative concentrations of hydroxide and alkoxide.

Saponification Irreversible

Although Reactions 2 and 3 are similar, an important difference is that saponification, unlike alcoholysis, is irreversible (10,11). However alcoholysis may be sent nearly to completion by any of several ways including product removal or, as is more frequently done, by a large excess of the alcohol ROH. In the latter case the alkoxide RONa is regenerated by Reaction 4 so that its concentration remains essentially unchanged whereas in saponification a loss in base occurs. This of course is the basis for the saponification value determination.

Reaction 4: R" ONa + ROH $\rightleftarrows$ R"OH + RONa

The Alcoholysis-Saponification System

Figure 1 summarizes the reactions described and illustrates the relationship between alcoholysis and saponification. The sole function or effect of water in this scheme is to supply hydroxide by the hydrolysis of alkoxide at A. In the absence of water, i.e., in an alkoxide solution prepared by dissolving metallic sodium in anhydrous alcohol, hydroxide formation and hence saponification will not occur. In the more likely case that some water will be present, the rate of alcoholysis will not be noticeably affected but an equivalent amount of saponification of the new ester will subsequently occur at C.

If the system (Reaction A, Fig. 1) is established by dissolving sodium hydroxide in anhydrous alcohol the fate of an ester (R'COOR") can be readily deduced. Since essentially all of the base is present as RONa alcoholysis, Reaction B, will proceed nearly to completion because of the high concentration of the alkoxide RONa which is maintained by Reaction D. Saponification by Reaction E will not occur to any appreciable extent both because of the low concentration of hydroxide and the rapidity with which alcoholysis is completed. Since the system was established by dissolving sodium hydroxide in the alcohol, however, sufficient water formed by Reaction A is present to send this reaction to the left as hydroxide is consumed by saponification of the new ester R'COOR at Reaction C. The final products are the same whether saponification occurs at E or via B and C. The foregoing is of no importance if the concern is the production of fatty acid soaps. If on the other hand fatty acid methyl esters are to be prepared from lipid esters a reexamination of some generally accepted procedures for accomplishing this is indicated.

EXPERIMENTAL PROCEDURE AND RESULTS

Methyl Esters by "Saponification-Reesterification"

An example of the "saponification-reesterification" method of methyl ester preparation is the widely used procedure of Metcalf et al. (12) which has been adopted by the AOAC (13). This procedure requires heating the oil in 0.5 M methanolic sodium hydroxide until solution is complete, a process normally requiring about 5 min. Saponification is presumed complete at that time. Methanolic BF_3 is then added and

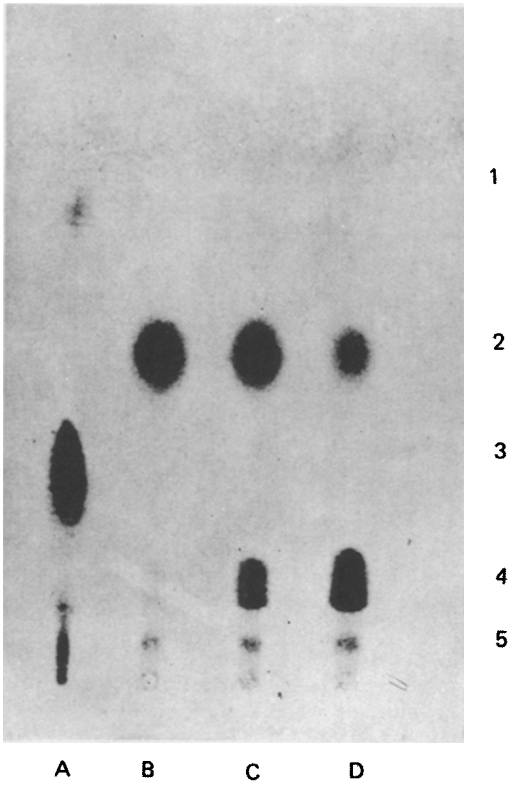

FIG. 2. The saponification of corn oil in boiling 0.5 M methanolic sodium hydroxide. Lane A, original corn oil; Lane B, 3 min; Lane C, 15 min; Lane D, 60 min. 1, steryl esters; 2, fatty acid methyl esters; 3, triglycerides; 4, fatty acids; 5, sterols.

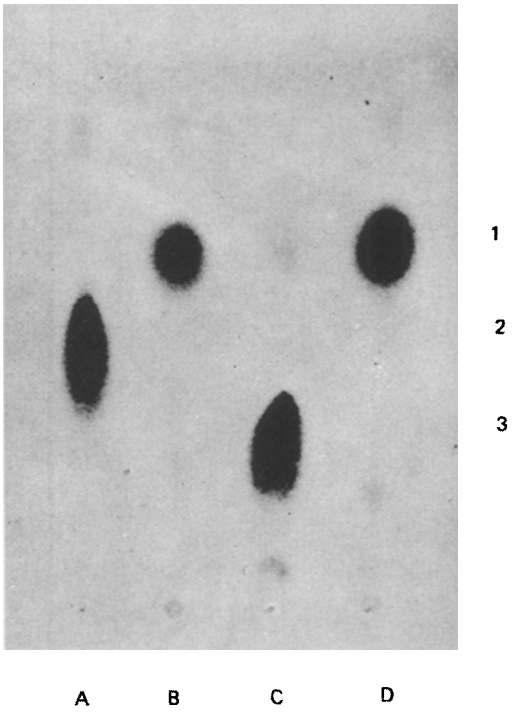

FIG. 3. The preparation of methyl esters from trilinolein by the "saponification-reesterification" procedure using 0.5 M methanolic sodium hydroxide containing 0.25% added water. Lane A, trilinolein; Lane B, 3 min; Lane C, 30 min; Lane D, 15 min base treatment followed by BF_3 treatment. 1, methyl linoleate; 2, trilinolein; 3, linoleic acid.

heating is continued for several minutes. The fatty acid methyl esters are then made ready for gas liquid chromatography (GLC) by any of several ways.

The progress of the saponification stage carried out as prescribed is shown in Figure 2. Corn oil, 200 mg samples, was boiled under reflux in 4 ml of 0.5 M methanolic sodium hydroxide. The reactions were stopped at various intervals by the addition of equivalent amounts of 2 M methanolic HCl. The several reaction mixtures were examined by thin layer chromatography (TLC) in the conventional manner using glass plates spread with 0.4 mm layers of Silica Gel H and the solvent system Skellysolve F, ethyl ether, acetic acid 85:15:1 v/v/v. The plates were charred after spraying with a sulfuric acid-sodium dichromate solution.

Figure 2 illustrates quite clearly that methanolysis has indeed preceded saponification and that the latter has proceeded only slowly. At 3 min, Lane B, methanolysis is nearly complete with only trace amounts of free fatty acids. Whether these arose from the saponification of glyceride or of methyl esters cannot be decided but the small amount of saponification makes the matter of little importance. At 15 min, Lane C, about 50% of the methyl esters have been saponified and at 60 min, Lane D, saponification was still not complete. Even at 120 min, not shown, detectable amounts of methyl esters remained. Identical results were obtained when potassium hydroxide was used as the base.

When carried to completion with methanolic BF_3 an excellent yield of methyl esters resulted (Fig. 3). To obtain the various mixtures shown, the reactions were carried out in a manner similar to those prevailing for the experiments shown in Figure 2 except that trilinolein was substituted for corn oil and 0.25% water was added to the methanolic sodium hydroxide to accelerate saponification. The reaction was stopped by the addition of methanolic HCl at 3 min (Lane B), 15 min (Lane C), or carried through the BF_3 treatment (Lane D). It is quite

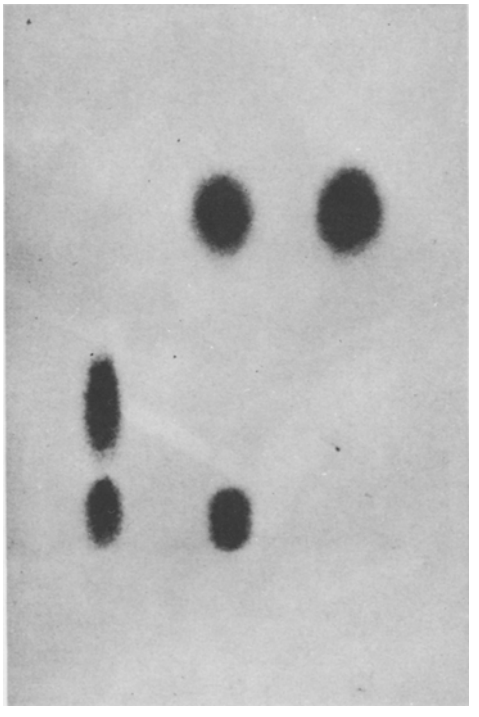

FIG. 4. The methanolysis and esterification of a trilinolein-linoleic acid mixture at room temperature (25 C). Lane A, original mixture; Lane B, neutralization of basic mixture at 3 min; Lane C, acidification for 30 min following 3 min basic treatment. 1, methyl linoleate; 2, trilinolein; 3, linoleic acid.

evident from Figure 3 that even in the presence of added water the sequence of events remains unchanged. Although the purity of the preparation obtained following BF_3 treatment is undeniable, there is no obvious improvement over that at 3 min. Since the "saponification-reesterification" process is actually a methanolysis-saponification-reesterification there is little advantage to be gained by proceeding beyond the methanolysis step. Indeed from Figure 2, where "anhydrous" methanolic sodium hydroxide was used, it appears that this is done anyway. The BF_3 treatment serves primarily to stop saponification and to "reverse" the little that may have occurred.

Precise timing of the neutralization was required to obtain the methyl ester preparation shown in Lane B, Figure 3. If the reaction were allowed to continue saponification would become increasingly evident. Such immediacy is generally undesirable. The need for neutralization can be virtually eliminated, however, by using moderate, i.e., ambient, temperatures and metallic sodium rather than sodium hydroxide to form methoxide. A mixture of 60 volumes of anhydrous methanol and 40 volumes of benzene made 1.0 M in metallic sodium will dissolve and methanolyze most lipid esters in minutes (a notable exception are steryl esters which require 15-20 min). Subsequent saponification in such a mixture is limited to the amount of water present and using reasonable precautions will become important only if very dilute solutions of lipids are used. Such a case is described below.

"Dimethoxypropane-Induced Transesterification"

The procedure of Mason et al. (14) requires the use, at room temperature, of a methanolysis mixture composed of 10 parts v/v of benzene, 4 of 2,2-dimethoxypropane (DMP) and 6 of methanol. The 20 ml reaction mixture containing 2 meq of sodium methoxide was used to methanolyze approximately 0.3 meq of glycerides. Following a 5 min reaction period when it was determined by GLC that methanolysis was 80% complete *and decreasing* the mixture was made acidic by the addition of methanolic-HCl. After 30 min the mixture was neutralized. Methanolysis was considered to have been "driven to completion" in the acid solution by the DMP.

An alternate explanation for these observations is that because of the large, approximately 7-fold, excess of base over ester, saponification of the dilute methyl esters was already apparent at 5 min. Since only about 5 mg of water, or 0.025%, would be necessary to supply sufficient hydroxide to saponify all of the methyl esters present this is entirely reasonable. The subsequent acid treatment would then perform the function not of "driving methanolysis to completion" but of re-forming the saponified methyl esters (Fig. 3).

The question is raised of any useful purpose being served by the acetal and our observations indicate that there are none since it would not serve as a water scavenger under basic conditions and water has little or no effect on methanolysis anyway. The work of Lorette and Brown (15) showed conclusively that the removal of water by DMP greatly increased the yield of methyl esters from the acid-catalyzed esterification, but these workers used a mixture comprised of nearly equimolar amounts of methanol, fatty acid or triglyceride, and the acetal. The reaction mixture of Mason et al. (14) contained an approximately 140:1 ratio of methanol-triglyceride fatty acid. The acidified equilibrium mixture would contain very little free fatty acid, as can be readily shown by TLC, in the presence of 1-2% water with or without acetal.

Preparation and Use of a Stable Methanolysis Reagent

A reagent capable of the rapid methanolysis of lipid esters at room temperature may be prepared as follows. Dissolve either 2.3 g of metallic sodium or 4.0 g of sodium hydroxide in 100 ml of a mixture consisting of 60 ml of anhydrous methanol, 40 ml of reagent grade benzene and 15 mg phenolphthalein. The reagent is stable indefinitely stored at room temperature unlike that of Mason et al. which must be prepared fresh each time. Similarly a reagent prepared replacing benzene with chloroform is effective but unstable because of the reaction of chloroform with the base.

The methanolysis of most lipid esters is accomplished simply by dissolving the lipid in the reagent to the desired concentration. A range of 1-10% is convenient for most purposes. The methanolysis of glycerides, phospholipids and wax esters is complete in 1-2 min. Steryl esters require 15-20 min. A more dilute, 0.25-0.50 N, reagent is as effective for glycerides and wax esters as is the described N reagent but the methanolysis of steryl esters is greatly retarded. The choice between sodium or sodium hydroxide is largely dictated by whether steryl esters are present in the mixture. Thus the convenience of using sodium hydroxide must be measured against the inevitable saponification that will occur subsequent to methanolysis. For this reason and regardless of the base used it is advisable to routinely neutralize the reaction mixture by the addition of methanolic-HCl. With the sodium reagent this may be done at almost any convenient time following addition of the base to the lipid. With the "sodium hydroxide" reagent, however, neutralization should be done within 10-15 min. In either case "reversal" of saponification is readily achieved by adding additional acid so that the final concentration is about 0.2 N. Esterification of the free fatty acids is essentially complete in 30 min so that they are not detectable on the gas chromatograph.

GLC analysis is done without further treatment of the reaction mixture with no damage to column or detector, and a sample may be introduced onto the chromatograph in less than 5 min from the start of the procedure.

Figures 4 and 5 show the results obtained when the described procedure, using sodium hydroxide, was applied to two mixtures, one consisting of trilinolein plus linoleic acid using a 0.5 M reagent, and the other trilinolein plus cholesteryl oleate using the 1.0 M reagent. The results shown in Figure 4 were obtained by neutralization at 3 min and acidification of a portion of the reaction mixture for an additional 30 min. To obtain the results shown in Figure 5 the basic reaction time was extended to 15 min. The mixture was then made slightly acid and immediately spotted for TLC. Some slight saponification is evident although in both experiments an obviously high purity of methyl esters resulted. Cholesterol does not interfere providing that chromatography is continued until it emerges from the column.

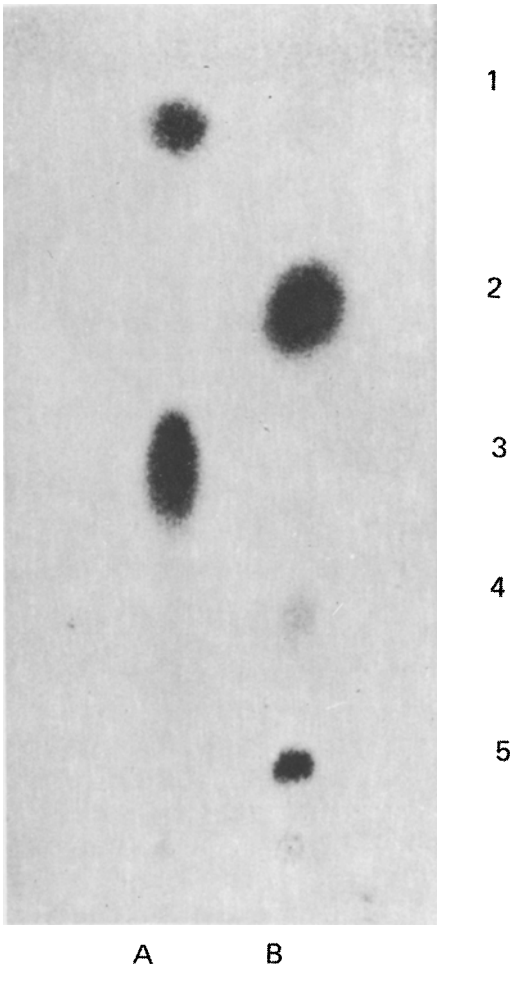

FIG. 5. The methanolysis of cholesteryl oleate and trilinolein at room temperature. Lane A, mixture trilinolein plus cholesteryl oleate; Lane B, reaction time 15 min in 1.0 M sodium methoxide. 1, cholesteryl oleate; 2, methyl esters; 3, trilinolein; 4, fatty acids; 5, cholesterol.

The two preceding experiments were both carried out using "sodium hydroxide" solutions. Since, as was established earlier, such solutions consist almost entirely of methoxide rather than hydroxide it follows that the water content of the reaction mixtures was very nearly that of the total base content or 0.9%

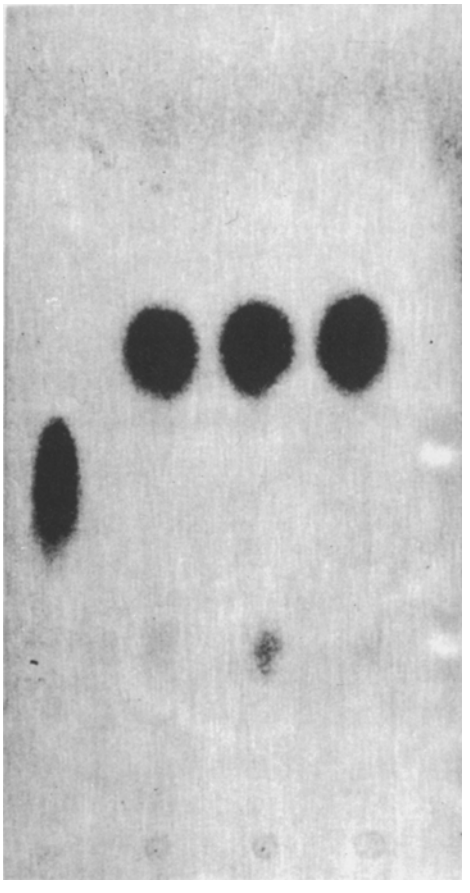

FIG. 6. Methanolysis at room temperature of trilinolein in the presence of approximately 2.4% water. Lane A, trilinolein; Lane B, 3 min reaction time; Lane C, 15 min reaction time; Lane D, 15 min reaction time followed by acidification for 30 min. 1, methyl linoleate; 2, trilinolein; 3, linoleic acid.

and 1.8% respectively. Identical results may be expected, then, from methoxide solutions prepared from metallic sodium and to which water is added to this extent. Such can readily be shown to be the case.

The water formed (cf., Reaction A, Fig. 1) when sodium hydroxide is dissolved in a benzene-methanol mixture imposes certain limits upon its composition. A phase separation occurs in a solution consisting of equal volumes of methanol and benzene if sodium hydroxide is dissolved to make a concentration in excess of about 0.6 M. No such difficulty is encountered with metallic sodium. Increasing the methanol content of the mixture permits an increase in the sodium hydroxide concentration. The primary effect of the presence of water is that of phase separation and not on either the rate of methanolysis or saponification. Thus water need not be considered if a single phase is obtained. This may be demonstrated as follows.

To 1.0 ml of a 10% solution of trilinolein in 1:1 methanol-benzene containing 2% water was added 0.5 ml of a 0.6 M solution of sodium hydroxide in methanol-benzene. The total water content of the reaction mixture was then 2.36%. The mixture was neutralized at 3 min by the addition of 5 M methanolic HCl (phenolphthalein). A similar reaction mixture was neutralized at 15 min. A portion, 100 μl, of this last solution was withdrawn to another tube and to it was added 15 μl of the methanolic HCl. At 15 min this was neutralized by titration with the "sodium hydroxide" solution. The various mixtures were then analyzed by TLC with the results shown in Figure 6.

As shown, methanolysis was complete at 3 min (Lane B) and saponification was barely in evidence. Saponification was more extensive at 15 min (Lane C) but cannot be considered excessive. It was effectively reversed by the acid treatment (Lane D). Although these results are admittedly qualitative it is significant that the small amount of free linoleic acid present in mixture C did not appear in subsequent analysis by GLC of the reaction mixture.

It has been proposed (16) that methanolysis in the presence of potassium methoxide occurs more rapidly than with sodium methoxide. We have been unable to substantiate this using TLC analysis and find no difference in the rate or the route by which the conversion of a triglyceride fatty acid to the potassium or sodium soap is accomplished. Only if the methanolysis is carried out in an essentially nonalcoholic medium, i.e., Skellysolve F (17), is there a noticeable difference and this is not one of rate but in the quantitative formation of crystalline monosodium glyceroxide while no such potassium compound is formed.

It is concluded that methyl ester preparation can be carried out rapidly and effectively at room temperature by the addition of a single stable reagent. If free fatty acids are present, either initially or as a result of saponification, these can be converted readily to the methyl esters by treatment with methanolic HCl at room temperature. It is suggested that undue emphasis has been placed upon the need for anhydrous conditions when carrying out methanolysis or esterification reactions. This has possibly resulted from a failure to properly appreciate: (1) that water is not directly involved in the saponification reaction; and (2)

that mass action considerations dictate that the acid catalyzed esterification of dilute solutions of fatty acids in methanol goes essentially to completion and that the equilibrium is not greatly upset by the addition of only several per cent of added water.

ACKNOWLEDGMENT

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Postnatal Changes in the Phospholipid Composition of Livers From Young Lambs

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ABSTRACT

The total lipids were extracted from the livers of newborn lambs, from the livers of lambs during the first week after birth and from the livers of adult sheep. After separation from the nonphospholipids on columns of silicic acid the phospholipids were analyzed by thin layer chromatography and quantitative gas liquid chromatography. In all samples phosphatidyl choline and phosphatidyl ethanolamine together accounted for about 80% of the total liver phospholipids. The phosphatidyl choline-phosphatidyl ethanolamine ratio in the livers of the newborn lambs was markedly less than the ratio in the livers of the adult sheep. Moreover there was a pronounced increase in the phosphatidyl choline-phosphatidyl ethanolamine ratio in the livers of the lambs during the first week after birth. In the liver phospholipids of the lambs the concentration of phosphatidyl inositol was lower and the concentrations of phosphatidyl serine and sphingomyelin were greater than the corresponding concentrations in the liver phospholipids of the adult sheep. It is proposed that the change in the phosphatidyl choline-phosphatidyl ethanolamine ratio in the livers of the lambs during the first week after birth is due, at least in part, to the marked change that occurs in the linoleic acid-arachidonic acid ratio in the tissues of the lamb during this period.

INTRODUCTION

Investigations into the lipid metabolism of the newborn lamb have shown that marked changes occur in the fatty acid composition of various tissues immediately after birth (1-4). The most pronounced and most significant changes in the fatty acid patterns were those involving the polyunsaturated fatty acids and recent work from this laboratory has indicated that the dietary requirements of essential fatty acids by the young lamb may well be considerably different from that of the young monogastric animal (3,4). In the liver the major changes in the polyunsaturated fatty acid pattern occurred in the phospholipid fraction and investigations into the fatty acid composition of the diacyl phospholipids of the livers of young lambs has provided information on the specific distribution of the fatty acids between these major phospholipid fractions (5). Preliminary results from these studies indicated that the fatty acid changes that occurred in the diacyl phospholipids of the lamb immediately after birth were possibly accompanied by pronounced alterations in the relative concentrations of the major phospholipid fractions. This possibility has been investigated in some detail and we now report results on the relative distribution of the major phospholipid fractions in the livers of lambs at birth and during the first week after birth. These results are compared with those obtained from adult sheep.

EXPERIMENTAL PROCEDURE

Lambs were obtained from a flock of pure-bred cheviot ewes. The diet and management of the ewes was similar to that detailed previously (3). The lambs were allowed to suckle and remain with the ewes during the experiment. Details of the lipid composition of ewes' milk during the first week of lactation have been given previously (6). Four lambs were killed immediately after birth before they had any opportunity to suckle. The remaining lambs were killed at intervals of 2,4 and 8 days after birth; three lambs were killed at each time interval. The lipids were extracted from the livers with chloroform-methanol 2:1 v/v by the method of Folch et al. (7). An estimate of the total dry matter content of the livers was obtained from the summation of the weight of total extractable lipid and that of the residue obtained after extraction with chloroform-methanol. The total phospholipids were separated from the nonphospholipids by fractionating portions of the lipid extracts on 3 g columns of silicic acid (100 mesh; A.R., Mallinckrodt Chemical Works, New York). As described by Moore and Doran (8) the nonphospholipids were eluted from the column with chloroform and the total phospholipids with chloroform-methanol 1:4 v/v. The individual phospholipids were then separated by fractionation on thin layer chromatoplates of silica

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gel (Camag A.G., Muttenz, Switzerland) with a solvent system composed of chloroform - methanol-acetic acid-water 25:15:4:2 v/v, as described by Skipski et al. (9). The fatty acids present in the major phospholipid fractions were converted into their corresponding methyl esters by the method of Stoffel et al. (10) and the relative concentrations of each of the major phospholipid fractions was determined by gas liquid chromatography in conjunction with the use of an internal standard as described by Christie et al. (11). The relative concentrations of each of the major phospholipid fractions were also estimated by determining the lipid phosphorus contents of the eluates by the method of Chen et al. (12). In all instances the recovery of the phospholipids from the thin layer chromatoplates was 95-105% of the total applied. These analytical procedures were also applied to the phospholipids isolated from the livers of four castrated male sheep of the cheviot breed, killed when 2 years old.

RESULTS AND DISCUSSION

The concentrations of total phospholipids in the liver tissues and the proportions of individual phospholipids expressed as weight percentages of the total phospholipids isolated from the livers of the lambs at birth and during the first week after birth are given in Table I together with the corresponding values for adult sheep liver. For comparison, values obtained by other investigators for the phospholipid composition of adult sheep liver are also included in Table I. In all of the samples analyzed phosphatidyl ethanolamine and phosphatidyl choline were the major phospholipids present and together accounted for about 80% of the total liver phospholipids. Phosphatidyl ethanolamine and phosphatidyl choline constituted 46% and 35% respectively of the liver phospholipids at birth, but in the liver phospholipids of the adult sheep the proportion of phosphatidyl choline far exceeded that of phosphatidyl ethanolamine. Our results for the phosphatidyl choline and phosphatidyl ethanolamine contents of the liver phospholipids of adult sheep are similar to those obtained in other laboratories (13,14). The relative proportions of phosphatidyl choline and phosphatidyl ethanolamine in adult sheep liver would appear to be similar to those reported for cow liver (15).

During the first week after birth there was a pronounced increase in the proportion of phosphatidyl choline and a pronounced decrease in the proportion of phosphatidyl ethanolamine in the liver phospholipids of the lambs, but even

TABLE I

Distribution of Individual Phospholipids in Livers From Lambs During First Week After Birth and From Adult Sheep[a]

	Lambs				Adult sheep			
Days after birth	0	2	4	8	b	c	d	e
Phospholipids at solvent front	2.4 ± 0.81	4.8 ± 0.20	6.0 ± 0.64	4.0 ± 0.32	3.2 ± 0.27	3.2	9.9	3.8	7.2
Phosphatidyl inositol	Trace	Trace	Trace	Trace	7.2 ± 1.03	6.1	9.9	6.3	8.2
Phosphatidyl ethanolamine	46.3 ± 2.09	41.2 ± 1.88	35.1 ± 4.56	34.7 ± 0.88	27.9 ± 1.66	26.5	28.9	14.1	27.9
Phosphatidyl serine	8.4 ± 0.25	12.0 ± 0.88	13.0 ± 0.65	9.2 ± 0.37	2.0 ± 0.07	4.4	3.5	1.3	2.3
Phosphatidyl choline	35.5 ± 2.25	37.0 ± 3.04	41.5 ± 0.87	47.8 ± 0.27	58.5 ± 1.15	55.2	47.4	54.6	39.7
Sphingomyelin	7.0 ± 0.75	5.0 ± 0.32	4.4 ± 0.83	4.3 ± 0.17	1.4 ± 0.26	4.2	8.1	2.3	5.0
Lysophosphatidylcholine	0.4 ± 0.11	Trace	Trace	Trace	---	---	1.9	---	---
Total phospholipid concentration (mg/g/dm in liver)	65 ± 2.07	94 ± 1.87	81 ± 2.34	90 ± 2.00	98 ± 1.62				

[a]Individual phospholipids are expressed as wt% of the total phospholipid (mean ± standard error).
[b]Results of Dawson (13).
[c]Results of Peters and Smith (14).
[d]Results of Getz et al. (19).
[e]Results of Scott et al. (20).

TABLE II

Weight Percentages of Fatty Acids in the Major Individual Phospholipids in Livers From Lambs During First Week After Birth

Fatty acid	Phosphatidyl ethanolamine			Phosphatidyl serine			Phosphatidyl choline			Sphingomyelin		
	At birth	8 Days after birth	SEM	At birth	8 Days after birth	SEM	At birth	8 Days after birth	SEM	At birth	8 Days after birth	SEM
16:0	19.4	10.8	±1.78	15.9	8.9	±4.20	39.4	24.1	±1.20	57.2	47.9	±3.04
16:1	3.5	0.2	±0.86	2.4	Trace	±0.85	4.9	0.5	±2.10	---	---	
18:0	16.4	28.9	±2.10	25.4	38.0	±3.56	13.5	25.2	±1.51	13.4	28.9	±1.98
18:1	23.2	19.5	±3.26	28.3	23.9	±4.25	31.8	27.8	±1.69	28.9	15.2	±1.64
18:2	1.3	8.4	±1.19	3.6	8.6	±1.24	0.7	12.9	±1.02	0.6	8.1	±1.01
18:3	Trace	1.1	±0.40	0.2	0.3	±0.36	0.3	1.9	±0.31	---	---	
20:3(*n*-9)	5.5	1.2	±1.10	7.1	2.1	±2.21	4.3	1.4	±0.91	---	---	
20:4(*n*-6)	9.8	10.4	±1.66	4.1	9.0	±2.26	2.2	3.8	±1.30	---	---	
22:5(*n*-3)	6.1	7.5	±1.72	4.6	4.8	±2.81	1.0	Trace	±0.89	---	---	
22:6(*n*-3)	13.6	10.1	±2.91	7.8	4.9	±2.74	1.5	Trace	±1.42	---	---	

on the eighth day after birth the values for these two major phospholipids had not reached the corresponding values observed for adult sheep liver. We could find no more than trace concentrations of phosphatidyl inositol in the liver phospholipids of the lambs, but in agreement with other investigators (see Table I) this phospholipid fraction was found to account for over 7% of the total liver phospholipids in adult sheep. Although there were no consistent or marked changes in concentration of phosphatidyl serine or sphingomyelin in the liver phospholipids during the first week after birth, the concentration of these two fractions of the liver phospholipids in young lambs were noticeably greater than the corresponding concentration in the liver phospholipids of adult sheep.

The most noteworthy features of the results in Table I are the marked differences between the phosphatidyl choline-phosphatidyl ethanolamine ratio in livers of the newborn lambs and that in livers of the adult sheep, and the progressive increase in this ratio that occurred in livers of the lambs during the first week after birth. These findings are consistent with those of Weinhold and Villee (16) and Biezenski et al. (17) who reported that the phosphatidyl choline-phosphatidyl ethanolamine ratio in livers of adult rats was considerably greater than that in livers of immature and newborn rats. It seems possible that this effect is due to developmental changes in the phospholipid content of the liver microsomes. In experiments with young rabbits Miller and Cornatzer (18) observed that during the first 14 days after birth there were no appreciable changes in the phosphatidyl choline-phosphatidyl ethanolamine ratios either in the liver mitochondria or in the liver microsomes; in addition there was no appreciable change in the total phospholipid content of the liver mitochondria. However during the same period there was a pronounced increase in total phospholipid content of the liver microsomes, the phosphatidyl choline-phosphatidyl ethanolamine ratio in which was markedly greater than that in the liver mitochondria. Getz et al. (19) have shown that in sheep liver also, the phosphatidyl choline-phosphatidyl ethanolamine ratio in the microsomes is greater than that in the mitochondria. It should be noted that although the work of Scott et al. (20) dealt only with various tissues from the ovine fetus, results obtained for the phospholipid composition of two fetal livers at about the 132nd day of gestation indicated a similar proportion of phosphatidyl ethanolamine to that found in the phospholipids obtained from a single sample of maternal liver.

In experiments with homogenates prepared from livers of rabbits of various ages, Baldwin and Cornatzer (21) noted a marked postnatal increase in the rate of synthesis of phosphatidyl choline from ^{14}C labeled cytidine diphosphate choline; there was little change in the rate of synthesis from ^{14}C labeled S adenosyl methionine. They proposed that this increase in the rate of synthesis via the CDP-choline dependent pathway might be due to an increased availability in the liver of 1,2-diglycerides containing linoleic acid. The work of Balint et al. (22) and Rytter et al. (23) had previously shown that phosphatidyl choline synthesized by the CDP-choline dependent pathway contained relatively high concentrations of linoleic acid, whereas that synthesized by the methylation of phosphatidyl ethanolamine contained relatively high concentrations of C_{20} and C_{22} polyunsaturated fatty acids. It has been noted by several investigators (24) that in most mammalian livers the phosphatidyl choline fraction tends to contain higher concentrations of linoleic acid and lower concentrations of C_{20} and C_{22} polyunsaturated fatty acids than does the phosphatidyl ethanolamine fraction. The distribution of fatty acids between the major phospholipid fractions in the liver of the newborn lamb during the first week after birth are given in Table II and their possible significance to the metabolism of phospholipids in the liver during this period has been fully discussed elsewhere (5). We have shown (25) that the whole carcass of the newborn lamb contains about 1000 mg of arachidonic acid but only about 300 mg of linoleic acid. However there is a marked increase in the linoleic acid content of the tissues during the first week after birth and the whole carcass of a 10-day-old lamb contains about 10,500 mg of linoleic acid but only about 3000 mg of arachidonic acid. This additional linoleic acid is derived from the milk of the ewe. It seems possible therefore that the pronounced change in the linoleic acid-arachidonic acid ratio in the tissues of the lambs during the first week after birth results in an analogous change in the fatty acid composition of the 1,2-diglycerides that become available for phospholipid synthesis in the liver. Such changes might be expected to promote the synthesis of phosphatidyl choline in the liver via the CDP-choline dependent pathway.

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Isomerization of L-Glyceraldehyde to Dihydroxyacetone During Glyceride Synthesis by Rat Liver Microsomes[1]

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ABSTRACT

L-glyceraldehyde is converted to phosphatidic acid by the action of rat liver microsomal enzymes and glycerol kinase in the presence of fatty acid, ATP, CoASH and NADH. L-glycerol 3-phosphate is not an intermediate in this synthesis since microsomes in the presence of NADH neither reduce L-glyceraldehyde nor, in the additional presence of glycerol kinase and ATP, convert it to L-glycerol 3-phosphate. However dihydroxyacetone is produced when L-glyceraldehyde is incubated with microsomes. This was shown enzymatically by the subsequent conversion to dihydroxyacetone phosphate which was confirmed by the oxidation of NADH in the presence of glycerol 3-phosphate dehydrogenase. Isomerization of L-glyceraldehyde and the synthesis of dihydroxyacetone phosphate may be one of several possible mechanisms in the conversion of the triose to either glucose or glyceride-glycerol which has been reported to occur in tissue.

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INTRODUCTION

In recent investigations of the synthesis of glycerides by particulate enzymes, DL-glyceraldehyde 3-phosphate (DL-GAP) has been used as a glyceride-glycerol precursor (1-5). It has been shown that the prior conversion of DL-GAP to dihydroxyacetone phosphate (DHAP) by microsomal triose phosphate isomerase is essential for glyceride synthesis (2-6). It is logical to expect L-GAP to be inert in this reaction since it is not a normal metabolite. In an attempt to confirm this supposition, we investigated glyceride synthesis using a system known to yield L-GAP and substituting it for DL-GAP. These studies have enabled us to show that rat liver microsomes cannot convert L-GAP to glycerides but that they can isomerize L-glyceraldehyde (L-GA) to dihydroxyacetone (DHA).

MATERIALS AND METHODS

DL-glyceraldehyde, dihydroxyacetone, GSH and CoASH were purchased from Sigma Chemical Co. The dimethylacetal of DL-glyceraldehyde 3-phosphate and the dimethylketal of dihydroxyacetone phosphate were also purchased from Sigma. The free carbonyl compounds were regenerated by hydrolysis with Dowex 50-X4,H^+ and their potassium salts were prepared by the addition of $KHCO_3$

TABLE I

^{32}P-Phosphatidic Acid Synthesis From L-Glyceraldehyde[a]

Flask	L-GA	DL-GA	NADH	HCPP	Phosphatidic acid synthesized (nmoles)	
1	+	-	+	-	28	30
2	-	+	+	-	31	35
3	-	-	+	-	3	3
4	+	-	-	-	3	3
5	+	-	+	-	23	23
6	+	-	+	+	20	18

[a]The reaction mixture contained L-GA (2 mM Flasks 1, 4-6), DL-GA (4mM, Flask 2), potassium palmitate (0.3 mM), γ-^{32}P-ATP (3mM), CoASH (0.067 mM), GSH (8mM), NADH (1.33 mM, except in Flask 4), $MgCl_2$ (3.3 mM), KF (8mM), glycerol kinase (40 μg protein), washed rat liver microsomes (1.2 mg protein), HCPP (0.26 mM, Flask 6) and potassium phosphate buffer pH 7.4 (133 mM) in a total volume of 1.5 ml. Incubations were carried out for 30 min at 37 C in a Dubnoff shaker and terminated by adding 5 ml chloroform-methanol 2:1 v/v. After acidification with 0.2 ml of 2N HCl, phosphatidic acid was isolated and quantitated by previously published procedures (7). The results of two separate experiments are given as nmoles of phosphatidic acid synthesized.

TABLE II

Conversion of L-Glyceraldehyde to Dihydroxyacetone Phosphate[a]

Flask	L-GA	Microsomes	nMoles NADH oxidized
1	+	+	188
2	-	+	0
3	+	-	4
4	-	-	10

[a]The incubation mixture contained L-glyceraldehyde (5 mM, flasks 1,3), ATP (1 mM), GSH (1 mM), glycerol kinase (25 μg protein), washed rat liver microsomes (4 mg protein, flasks 1,2), potassium phosphate buffer, pH 7.4 (50 mM) in a volume of 2 ml. After incubation for 30 min at 37 C in a Dubnoff shaker, the reaction mixture was centrifuged at 104,000 x g for 30 min. To the supernatant, NADH (0.166 mM) and glycerol 3-phosphate dehydrogenase (30 μg protein) were added. The volume was adjusted to 3 ml by the addition of water and after incubation at 37 C for 10 min, the optical density was measured at 340 mμ in a Gilford Spectrophotometer; nmoles of NADH oxidized was calculated using the molar absorption coefficient, 6.2 x 10^6 Cm^2/mole.

before use. L-glyceraldehyde was purchased from Fluka AG; NADH, ATP and crystalline glycerol 3-phosphate dehydrogenase from Calbiochem; glycerol kinase was obtained from Boehringer Mannheim Corporation and 1-hydroxy-3-chloro-2-propanone phosphate was a gift from F.C. Hartman, Oak Ridge National Laboratory, Oak Ridge, Tenn. The 1-^{14}C-palmitic acid purchased from Volk Radiochemical Co. was purified before use by thin layer chromatography (>99%); unlabeled purified palmitic acid was from Hormel Institute. The γ-^{32}P-ATP was purchased from Amersham Searle Corp.

Rat liver microsomes were isolated from a sucrose homogenate and washed according to previously published procedures (5). Incubations were carried out as described in the tables. Freshly prepared solutions of L-GA were used in all experiments. For the determination of glyceride synthesis, the incubations were terminated by the addition of 5 ml of chloroform-methanol 2:1 v/v. After acidification with 0.2 ml of 2N HCl, phosphatidic acid was isolated and quantitated by the method of Johnston et al. (7). Glycerol and L-glycerol 3-phosphate were detected by the production of NADH from NAD^+ under the assay conditions for glycerol kinase described by Kennedy (8). The presence of dihydroxyacetone was determined following its conversion to DHAP by a 30 min incubation with glycerol kinase (25 μg protein), ATP (1 mM), GSH (1 mM) and potassium phosphate buffer (pH 7.4, 50 mM). Dihydroxyacetone phosphate production was confirmed by the oxidation of NADH in the presence of crystalline glycerol 3-phosphate dehydrogenase and NADH under conditions described by

TABLE III

Isomerization of L-Glyceraldehyde to Dihydroxyacetone by Hepatic Microsomes[a]

	Additions		
Flask	L-GA	Microsomes	nMoles NADH oxidized
1	+	+	280
2	+	-	2
3	-	+	0
4	+	+[b]	58
5	+	-	0

[a]The reaction mixture contained L-glyceraldehyde (5.7 mM, flasks 1, 2, 4, and 5), washed rat liver microsomes (4 mg protein in flasks 1, 3, and 4), bovine serum albumin (4 mg in flask 5) and potassium phosphate buffer pH 7.4 (57 mM) in a total volume of 1.75 ml. After incubating at 37 C for 30 min the mixture was centrifuged at 104,000 x g for 30 min. To the supernatant was added glycerol kinase (25 μg protein), ATP (1 mM) and GSH (1 mM) in a volume of 2 ml and incubated for 30 min at 37 C. Incubation was continued for an additional 10 min after the addition of NADH (0.166 mM), glycerol 3-phosphate dehydrogenase (30 μg protein) and adjustment of the volume to 3 ml. At the end of the incubation optical density was determined at 340 mμ in a Gilford spectrophotometer; nmoles of NADH oxidized was calculated using the molar absorption coeffocient, 6.2 x 10^6. Cm^2/mole.

[b]Heat inactivated at 100 C for 10 min.

TABLE IV

Effect of HCPP on the Microsomal Isomerization of L-Glyceraldehyde to Dihydroxyacetone

	Additions			
Flask[a]	L-GA	DL-GAP	HCPP	nMoles NADH oxidized
1	+	-	-	169
2	+	-	+	114
3	-	+	-	191
4	-	+	+	4

[a]Flasks 1 and 2: Incubation conditions were identical to Flask 1, Table III except for the following: Microsomes (1.5 mg protein was used). HCPP (0.2 mM) was present in Flask 2 during the incubation of L-GA and microsomes. Flasks 3 and 4: Reaction mixture contained DL-GAP (3 mM), HCPP (0.2 mM, Flask 4), potassium phosphate buffer pH 7.4 (50 mM) and washed rat liver microsomes (1.5 mg protein) in a total volume of 2 ml. After incubation for 30 min at 37 C, it was centrifuged at 104,000 x g for 30 min. To the supernatant (0.25 ml) was added NADH (0.166 mM), glycerol 3-phosphate dehydrogenase (20 μg protein) and water to make the final volume 3 ml. Incubation was carried out for an additional 10 min at 37 C and the optical density at 340 mμ was measured; nmoles of NADH oxidized was calculated from the molar absorption coefficient of NADH, 6.2 x 10^6 Cm^2/mole.

Beisenherz et al. (9). Protein determination was carried out by the procedure of Lowry et al. (10).

RESULTS AND DISCUSSION

Our earlier studies demonstrated the synthesis of ^{32}P-phosphatidic acid from dihydroxyacetone by the combined action of microsomal enzymes and glycerol kinase in the presence of γ-^{32}P-ATP, NADH, CoASH and potassium palmitate (5). We extended these studies employing L- or DL-glyceraldehyde since glycerol kinase is known to act not only on glycerol and DHA but also on L-GA (11-14). If L-GAP were converted to glycerides via its isomerization to DHAP, ^{32}P-phosphatidic acid should be produced. Furthermore inculsion of 1-hydroxy-3-chloro-2-propanone phosphate (HCPP), an irreversible inhibitor of triose phosphate isomerase (15-17), might completely inhibit this synthesis since, in earlier studies, conversion of DL-GAP to glycerides was not observed in the presence of HCPP (2-5). The results of these experiments are given in Table I. When L- or DL-GA is present in the reaction mixture, phosphatidic acid synthesis occurs (Flasks 1 and 2). The reaction required the triose and NADH since in their absence only minimal glyceride synthesis was observed (Flasks 3 and 4). Due to the presence of glycerol kinase and ATP, L-GA can be converted to L-GAP during the course of incubation. However it is likely that this triose phosphate does not serve as a precursor of glycerides. This was shown by separate experiments in which incubations of L-GA, γ-^{32}P-ATP, GSH and glycerol kinase were carried out to produce L-GAP. Subsequently the kinase was heat inactivated and following the addition of palmitate, ATP, CoASH, NADH and microsomes, the reaction was continued. These experiments failed to yield ^{32}P-phosphatidic acid. That L-GAP does not serve as a glyceride-glycerol precursor was further supported by the observation that appreciable phosphatidic acid synthesis occurred even in the presence of HCPP (Flask 6). HCPP used in these experiments was active because, when DL-GAP and 1-^{14}C-palmitate were incubated with microsomes, ATP, CoASH, and NADH as described earlier (4,5), complete inhibition of glyceride synthesis was observed.

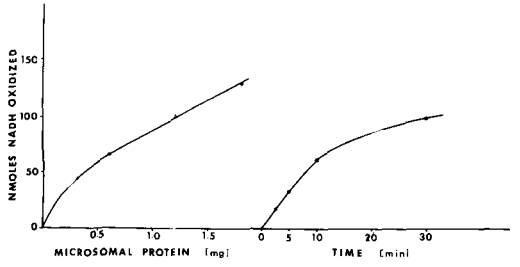

FIG. 1. Effect of microsomal protein and time of incubation on the formation of DHA from L-GA. Incubations were performed with L-GA and varying amounts of microsomal protein for 10 min or with L-GA and a constant amount of microsomal protein (0.6 mg) for varied time intervals. The reaction for specific time intervals was terminated by the addition of 0.5 ml of 5% $HClO_4$ and centrifuged at 5,000 x g for 5 min. The pH of the supernatant was adjusted to 7.0 by the addition of $KHCO_3$. DHA formation was measured by nmoles of NADH oxidized as described in Table III.

These studies suggested that L-GA may have been converted to phosphatidic acid by a mechanism that does not involve its phospho-

rylation. Two mechanisms may explain our results. First, L-GA may have been reduced to glycerol by a microsomal dehydrogenase in the presence of NADH and then by the action of glycerol kinase converted to L-glycerol 3-phosphate, which may have been the direct acyl acceptor. This possibility is unlikely because L-GA dependent oxidation of NADH was not observed in the presence of microsomes and glycerol was not produced. Also, incubations of L-GA with ATP, NADH, microsomes, and glycerol kinase did not yield L-glycerol 3-phosphate.

A more probable explanation of our results is that L-GA may have been isomerized to DHA and further converted to DHAP by the action of glycerol kinase and ATP. Recent investigations have shown that dihydroxyacetone phosphate can serve as a direct glyceride-glycerol precursor (2-5,18). Furthermore glyceride synthesis from DHAP has been known to be unaffected by the presence of HCPP (2,3,5). That this is indeed the effective mechanism is shown by the results given in Table II. When potassium palmitate, CoASH and NADH were excluded from the reaction mixture for phosphatidic acid synthesis, DHAP was produced as evidenced by the oxidation of NADH in the presence of glycerol 3-phosphate dehydrogenase (Flask 1). The production of DHAP requires L-GA and microsomes (Flasks 2-4). These studies were extended with L-GAP by first incubating L-GA with ATP, GSH and glycerol kinase. Following the heat inactivation of the kinase, the reaction was continued in the presence of microsomal protein. As would be expected, DHAP was not produced in these experiments suggesting that the microsomal triose phosphate isomerase is inactive toward L-GAP.

The capacity of the microsomes to isomerize L-GA to DHA was further investigated by the experiments given in Table III. L-GA was isomerized to DHA by incubation with microsomes (Flask 1). In the absence of microsomes, the isomerization of L-GA was not observed, indicating that it was free from DHA (Flask 2). Furthermore the DHA formation is dependent on the presence of L-GA (Flask 3). Not all of the microsomal activity which isomerizes L-GA is heat-labile (Flask 4). Several experiments failed to demonstrate complete destruction of this activity in spite of the heat inactivation of microsomal protein at 100 C for 10-20 min. However isomerization of L-GA was always significantly greater when native microsomes were employed. DHA was not produced when microsomal protein was replaced by bovine serum albumin (Armour and Co.) in the reaction mixture (Flask 5). The rate of isomerization of L-GA was found to be proportional to the microsomal protein concentration and was linear during the initial 10 min of incubation (Fig. 1).

Appreciable synthesis of phosphatidic acid from L-GA even in the presence of HCPP, observed in the experiment reported in Table I, and the isomerization of L-GA during this process would suggest that the inhibitor has no significant effect on the production of DHA from L-GA by microsomes. This is supported by the results of experiments shown in Table IV. The isomerization of L-GA occurred to an appreciable degree in the presence of HCPP (Flasks 1 and 2). However, as has been observed with preputial gland tumor microsmes (3), rat liver microsomes also effectively inhibited the microsomal triose phosphate isomerase (Flasks 3 and 4). It is likely that the microsomal activity that isomerizes L-GA is distinct from the particulate triose phosphate isomerase.

Previous investigations have shown the conversion of L-GA to either glycogen or glycerol of triglycerides employing rat liver slices and segments of epididymal fat pads respectively (19,20). Since this triose can be phosphorylated to L-GAP for which no further metabolism is known, it has been thought that it may be reduced to glycerol and phosphorylated to L-glycerol 3-phosphate which could then serve as a precursor for the synthesis of either glucose or triglycerides. While these reactions are possible in the cells, other mechanisms may also exist. Thus, following the use of 3-^{14}C-L-GA, the recovery of appreciable ^{14}C activity in the 1-C of triglyceride glycerol has led Antony et al. to suggest several possible alternate routes (20). One of these is the isomerization of L-GA to DHA. Our previous studies on phosphatidic acid synthesis employing DL-GAP (4,5), the investigations presented in this communication using L-GA, and the nature of the inhibitory effects of HCPP on the glyceride synthesis also suggested a possible isomerization of L-GA to DHA. The results of our incubations of L-GA with rat liver microsomes demonstrate that such an isomerization to DHA does occur. In cells, DHA so produced may be phosphorylated by the action of glycerol kinase to yield DHAP which could act as glyceride-glycerol precursor either directly (2-5,18) or subsequent to its reduction to glycerol 3-phosphate (21) and could be the source of glycogen also.

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Structural Comparison Between Triglycerides and Phospholipids From Pig Kidney

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ABSTRACT

The compositional specificity of the major diacyl phosphatides, plasmalogens and triglycerides of pig kidney has been determined. The triglycerides have been shown to be esterified predominantly with saturated fatty acids in the 2 position while the phosphatides have predominantly unsaturated fatty acids in this position. Such complete "inversion" of the structure of triglycerides and phospholipids is not normally seen in mammalian tissue and suggests that the synthesis of structural polar lipids involves other steps than the reaction of a diglyceride with CDP-ethanolamine or CDP-choline. This marked difference in the fatty acid distribution of the polar lipids and triglycerides of the pig kidney may render this tissue especially suitable for studies on the turnover and synthesis of structural lipids.

INTRODUCTION

It is well recognized that the fatty acids esterified in the 2 position in mammalian triacyl-*sn*-glycerols and diacyl-*sn*-phosphatides are predominately long chain unsaturated acids (1). Such similarities between the chain composition of these lipids are in agreement with the contention that diglycerides are the precursors of both lipids (2).

That difference exists between the composition of the fatty acid esterified to the 2 position of triglycerides and phosphatides is attributed to enzymic activities acting predominantly on the fatty acid esterified to the 2 position of the phosphatides (3).

In pig tissue, however, the composition of the fatty acids esterified to the 2 position of the triglycerides has been shown to be mostly saturated fatty acids (4), while no data are available on the compositional specifics of the esterified fatty acids of pig tissue phosphatides. The aldehydogenic chain of pig tissue plasmalogens (alk-1'-enyl-acyl glyceryl ethers) has been shown to be mostly saturated (5), and to occupy the 1 position of the phosphatides (6), a compositional and speriospecificity normally seen in mammalian tissues (7).

These observations led us to investigate the compositional specificity of the fatty acids esterified in the 2 position of pig tissue phosphatides in relation to the fatty acids esterified in the same position of the triglycerides, and the composition specificity of the aldehydogenic linked chains of the plasmalogens in relation to that of the diacylphosphatides. This investigation was undertaken as part of a major study on structural relationships between classes of lipids, their interrelationships and biosynthesis.

MATERIALS AND METHODS

Materials

Methyl esters of palmitic, stearic, oleic, linoleic, linolenic and arachidonic fatty acids (The Hormel Institute, Austin, Minn.) were used for the identification of gas liquid chromatography (GLC) peaks of methyl esters prepared from sample fractions. A portion of the methyl ester standards was reduced with $LiAlH_4$ (8) and the resulting alcohols were acetylated (9). These alcohol acetates served as standards for the identification of GLC peaks of the alcohol acetates prepared from sample fractions. Pancreatic lipase was purchased from Nutritional Biochemicals Corporation and used without further purification. Phospholipase A (Crotalus Atrox Venom) was purchased from Allens Reptile Institute.

Extraction and Initial Separation of Tissue Lipids

Pig heart, kidney, spleen and brain were obtained from a local slaughterhouse. The organs were rapidly cooled. Connecting tissue and deposit fat were carefully removed. About one-half of the organs were cut into small pieces and homogenized in a Waring Blendor with chloroform-methanol 2:1 v/v. Further homogenization was achieved by using a Potter-Elvehjem homogenizer. The homogenized tissue was filtered and the chloroform-methanol was removed from the filtrate under vacuum. The lipids were then extracted from the aqueous suspension by the method of Bligh and Dyer (10). The total lipids were separated into neutral and phospholipid fractions by use of a short silicic acid column (11). The lipids were stored in chloroform at -23 C until used.

The methods used for separation and deter-

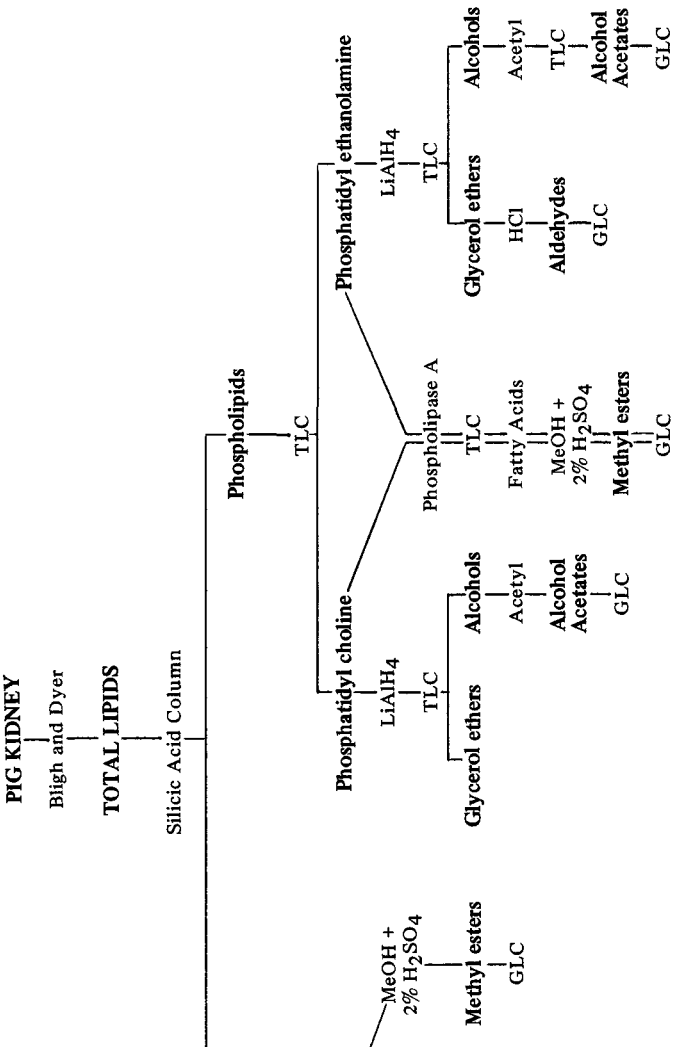

FIG. 1. Flow diagram of isolation and analytical procedures.

mination of lipid classes are shown systematically in Figure 1.

Separation of Lipid Classes by Thin Layer Chromatography (TLC)

Approximately 10 mg of total neutral lipids were applied in a band on a 20 x 20 cm glass plate coated with 0.25 mm Silica Gel G. The plates were developed in a solvent system of hexane-diethylether-acetic acid 80:20:1 v/v/v. After development the plates were sprayed with a solution of 0.1% 2,7-dichlorofluorescein in ethanol and viewed under UV light to locate the bands. The sterol ester band was scraped directly into a tube containing 2-3 ml of 2% H_2SO_4 in methanol. The tube was blanketed with nitrogen, sealed and heated for one hour at 100 C to form the methyl esters of the fatty acids. The recovered products were analyzed by GLC without removal of the free cholesterol since cholesterol is not eluted under the GLC conditions used.

The triglycerides were eluted from the silica gel on a sintered glass funnel with diethylether. The solvent was removed and a small portion of the triglycerides trans-esterified directly for determination of total acyl groups. The remaining triglycerides were submitted to pancreatic lipase hydrolysis (12).

The phospholipids were separated by TLC on HRB Silica Gel plates using a solvent system of chloroform-methanol-acetic acid-water 50:25:8:4 v/v/v/v. Only the phosphatidylethanolamine (PE) and phosphatidylcholine (PC) bands were recovered. The elution of the sample from the silica gel was carried out as previously described (13). The purity of the isolated fractions was determined by TLC against known standards.

Enzyme Hydrolysis of Triglycerides and Phospholipids

The pancreatic lipase hydrolysis of the triglycerides was carried out essentially according to the procedure of Mattson and Volpenhein (12) with appropriate scaling down to accommodate the smaller sample size. The hydrolysis products were recovered by three hexane-diethylether 1:1 v/v extractions of the acidified (HCl to pH 2) hydrolysate. The hydrolysis products were separated by TLC on Silica Gel G using a hexane-diethyl ether-acetic acid 50:50:2 v/v/v system. The developed plates were sprayed with 2,7-dichlorofluorescein (0.2% in ethanol), viewed under UV light and the monoglyceride band scraped directly into a tube and esterified in the same manner as the sterol esters.

The isolated phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were treated with phospholipase A (Crotalus Atrox Venom, Allens Reptile Institute) as described previously (14).

About 19 mg of either phosphatide were incubated with 2 mg snake venom. The venom was dissolved in 2 ml 0.2 M Tris-HCl pH 8.0, and kept in a boiling water bath for 10 min. After cooling, this suspension was added to the reaction mixture composed of either phosphatide (19 mg), sodium deoxycholate (5 mg), calcium chloride 45% (0.1 ml) and diethylether (0.5 ml). The reaction was carried out at room temperature under vigorous shaking overnight. The reaction mixture was acidified with hydrochloric acid to pH 2 and the liberated fatty acids extracted with hexane-diethylether 1:1 v/v. The fatty acids were then isolated by preparative TLC on Silica Gel G in a solvent system of hexane-diethylether-acetic acid 50:50:1 v/v/v to remove any long chain fatty aldehydes which may have been liberated from the plasmalogens during the acidification.

The fatty acid band was scraped directly into a tube and esterified as described earlier.

TABLE I

Glyceryl Ether Content[a] of Pig Kidney Phosphatidylethanolamine[b] and Phosphatidylcholine

Phosphatide	O-Alkyl	Alk-1'-enyl
PE[c]	Trace	16.9 ± 1.0
PC[d]	0.8	1.9

[a]Values expressed as weight per cent of the recovered $LiAlH_4$ reduction products. This value should be multiplied by 2 to obtain an approximate value for ether-acyl forms.

[b]See text for percentage plasmalogen in pig kidney phosphatidylethanolamine as determined by other methods.

[c]Duplicate determination on three samples.

[d]Duplicate determination on single samples.

Quantitation and Analysis of Glyceryl Ethers

A portion of the PC and PE was reduced with $LiAlH_4$ and the content of glyceryl ethers determined by photodensitometry of the TLC separated products (15). The total $LiAlH_4$ reduction products were separated by TLC using diethylether-water 100:0.5 v/v as the developing solvent. The alcohol bands (derived from the reduction of the esterified acids) and the alk-1'-enyl glycerol ether band from the phosphatidylethanolamine were recovered. The alcohols were eluted from the silica gel using 20% methanol in dietlylether and acetylated (9). The acetylated alcohols were purified by TLC using hexane-diethylether 90:10 v/v as the developing solvent. Their composition was de-

TABLE II

The Aldehydogenic Chain Composition (Wt %) of Pig Tissue Polar Lipid Plasmalogens[a]

Fatty aldehyde[b]	Heart	Spleen	Brain	Kidney	PE[c]	PE[d]
14:0	—	Trace	—	1.7	1.5	1.3
15:0	Trace	Trace	—	1.8	1.5	1.3
16:0	51.0	42.8	18.7	68.6	65.3	68.5
16:1	Trace	2.0	0.6	3.0	1.9	—
17:0	3.9	4.7	1.2	3.6	3.4	2.6
18:0	26.0	30.8	31.6	13.1	16.3	25.6
18:1	17.3	16.3	47.3	7.2	7.5	—
19:1 + 18:2	1.8	2.7	0.6	1.0	0.8	—
20:0	Trace	0.7	Trace	Trace	0.7	0.6
20:1 + 18:3	Trace	Trace	Trace	Trace	1.1	—
Saturated	80.9	79.0	51.5	88.8	88.7	
Unsaturated	19.1	21.0	48.5	11.2	11.3	

[a]Determined after acid hydrolysis of the total polar lipids.

[b]Identified by the shorthand system of nomenclature according to Farquhar et al. (33).

[c]Determined after acid hydrolysis of the 0-alk-1'-enyl glycerol ethers obtained by $LiAlH_4$ reduction of pig kidney phosphatidylethanolamine.

[d]Determined after hydrogenation as the isopropylidene derivative of pig kidney phosphatidylethanolamine reduced with $LiAlH_4$.

termined by GLC after elution from the silica gel with 20% methanol in diethylether. Part of the alk-1'-enyl glycerol ether band was scraped directly into a tube and hydrolyzed as described by Anderson et al. (16). The other portion of the alk-1'-enyl glycerol ethers was eluted from the silica gel in the same manner as the alcohols and hydrogenated in the presence of Adams catalyst. A portion of the resulting saturated 0-alkyl glycerol ether was converted to isopropylidene derivatives (17) and analyzed by GLC as a check on the direct hydrolysis of the alk-1'-enyl ethers. Another portion of the 0-alkylglycerol ethers was used to determine their isometric form by the use of sodium aresnite-impregnated silica gel thin layer plates as previously described (18).

Gas Liquid Chromatography

All GLC analyses were carried out with a Model 4000 Victoreen, dual column hydrogen flame detector unit. The instrument was fitted with two 6 ft x 1/8 in. columns packed with 10% EGSS-X on 100-120 mesh Gas-Chrom P (Applied Science Laboratories, State College, Pa.). A carrier gas (He) flow of 30-35 ml/min and injection port and detector temperatures of 260 C were maintained in all analyses. The aldehydes, methyl esters, alcohol acetates, and isopropylidenes of 0-alkyl glycerol ethers were analyzed at column oven temperatures of 175, 185, 190 and 200 C respectively.

RESULTS AND DISCUSSION

Content and Composition of Glyceryl Ethers

The percentage distribution of the polar to nonpolar lipids varies considerably in the pig tissues investigated. In heart tissue 85% of the total extractable lipids are polar lipids; in kidney, only 57% are polar lipids while liver, spleen and brain lipids are composed of about 70% polar lipids. The content of alkyl and alk-1'-enyl glyceryl ethers in the phosphatidylcholine and phosphatidylethanolamine fractions of pig kidney lipids is shown in Table I. Only trace amounts of alkyl glyceryl ethers are present in the phosphatidylethanolamine fraction while about 34% of this fraction was shown to be alk-1'-enyl-acyl phosphatidylethanolamine. In the phosphatidylcholine fraction, about 2% is alkyl-acyl-, and 4% alk-1'-enyl-acyl species. If the proposed (19) pathway for the synthesis of alk-1'-enyl acyl phosphatides through the alkyl ether as an intermediate is correct, the large difference in the ratio of alkyl to alk-1'-enyl phosphatides in this tissue postulates considerable difference in the metabolic stability of the phosphatidylethanolamine and phosphatidylcholine species, or a considerable specificity of the "alkyl dehydrogenase" (19).

The 0-alk-1'-enyl glycerol obtained by $LiAlH_4$ reduction of the phosphatidylethanolamine ran on argentation TLC after hydrogenation with an R_f value identical to that of 1-0-alkyl-glycerol ether. The ethanolamine plasmalogen of pig kidney most likely, therefore, has the isomeric configuration of 1-alk-1'-enyl-2'-acyl-*sn*-glyceryl ethanolamine. This configuration is the only plasmalogen structure detected in both mammalian (7) and bacterial lipids (20).

The percentage alk-1'-enyl-acyl phosphatidyl-

TABLE III

Fatty Acid Composition (wt %) of Pig Kidney Lipid Classes

Fatty acid	Total TG	2 Position TG	Total PC	2 Position PC	Total PE	2 Position PE	Calc[a] PE	Sterol esters
14:0	1.1	2.5	—	Trace	—	—	0.3	1.3
15:0	—[b]	—	Trace[c]	Trace	Trace	Trace	0.3	1.8
16:0	26.2	62.9	23.1	14.0	5.0	1.1	18.7	18.5
16:1	1.7	2.8	1.1	0.8	0.4	Trace	0.7	4.5
17:0	0.8	1.2	1.0	0.5	0.7	Trace	1.3	Trace
18:0	21.3	6.1	16.3	4.4	15.4	1.0	15.6	8.9
18:1	36.2	16.9	18.3	20.2	12.3	5.3	11.2	26.1
18:2	9.5	6.1	15.3	21.8	6.4	6.3	5.1	19.6
18:3	1.5	0.6	0.7	0.6	0.6	0.4	0.7	2.9
20:0	Trace	Trace	—	—	—	—	0.2	0.7
20:2	0.7	Trace	1.3	1.4	0.6	0.3	0.5	2.0
20:3	Trace	Trace	2.9	3.9	2.1	2.2	1.6	1.7
20:4	1.0	0.9	17.0	27.8	49.4	75.7	38.2	6.9
20:5	—	—	Trace	Trace	Trace	Trace	Trace	2.2
A[d]	—	—	1.5	2.2	4.2	3.8	3.3	2.9
B[d]	—	—	Trace	Trace	Trace	Trace	Trace	Trace
C[d]	—	—	Trace	1.1	Trace	Trace	Trace	Trace
D[d]	—	—	1.5	1.4	2.8	3.9	2.2	Trace
Saturated	49.4	72.7	40.4	18.9	21.1	2.1	36.4	31.2
Unsaturated	50.6	27.3	59.6	81.2	78.8	97.9	63.5	68.8

[a]Calculated by inclusion of the alk-1'-enyl glyceryl ether side chain as acyl assuming 45.4% alk-1'-enyl-acyl phosphatidyl ethanolamine.

[b]Not detected.

[c]Trace = <1%.

[d]A, B, C and D retention times correspond closely to 22:3, 22:4, 22:5 and 22:6.

ethanolamine of the ethanolamine phosphates obtained by the $LiAlH_4$ reduction procedure of Wood and Snyder (8) reported here is about 11% lower than that reported by Gray and MacFarlane (5). Similar discrepancies between data obtained by quantitation of phosphatide plasmalogens using the $LiAlH_4$ reduction procedure and other methods have previously been reported by us (11). Therefore we carefully evaluated the plasmalogen content of pig kidney phosphatidylethanolamine by several methods. When the plasmalogen content of this fraction was determined by the dimethylacetal procedure described by Gray (21), the alkaline hydrolyses procedures of Tarlov and Kennedy (22) and Hajra et al. (23), and the colorimetric procedure of Williams et al. (24), we obtained values of 45.0%, 44.8%, 45.8% and 45.9%, respectively. These results clearly indicated a considerable discrepancy between the results obtained by $LiAlH_4$ reduction of phosphatidylethanolamine and the other more conventional methods for plasmalogen determination. The $LiAlH_4$ reduction procedure is rapid and convenient, but we feel extreme caution must be exercised in interpretating quantitative and possible qualitative data obtained by this procedure.

The composition of the aldehydogenic chain of the total lipid plasmalogens is shown in Table II. Also in this table the aldehydogenic chain composition of the pig kidney phosphatidylethanolamine is shown. In one experiment these chains were liberated by hydrochloric acid hydrolyses (16) and the aldehydes determined by GLC. In another experiment the 0-alk-1'-enyl glycerols were obtained by $LiAlH_4$ reduction, hydrogenated, and their chain composition determined as their isopropylidene derivative. These two procedures give compatible results. Our results indicate that considerable variations exist in the aldehydogenic composition of various pig tissue plasmalogens. The ratio of saturated to unsaturated aldehydes ranges from 1.06 in pig brain tissue to 7.91 in pig kidney tissue. The aldehydic chains of pig spleen and pig heart tissue phosphatides were also found to be mostly saturated, with a ratio of 3.61 and 4.31, respectively. Similar results have been reported by Gray and MacFarlane (5) for pig spleen, lung and kidney. However our results indicated only trace amounts of vinyl ether linked branched chains, while they reported considerable amounts of these chains in pig kidney plasmalogens. We find that about 90% of the aldehydogenic linked chains of the pig kidney plasmalogens are saturated fatty aldehydes, which is a considerably higher per cent saturation of these chains than seen in other mammalian plasmalogens (7).

Comparison of Total Acyl Groups and 2 Position Acyl Groups in Triglycerides, Phosphatidylethanolamine and Phosphatidylcholine

The fatty acid composition of the acyl groups esterified to triglycerides, phosphatidylethanolamine and phosphatidylcholine of pig kidney lipids is shown in Table III. The determinations of the total acyl groups of the phosphatides were performed on the acetate derivatives of the purified long chain alcohols obtained after $LiAlH_4$ reduction of the lipids. These values therefore include the ester-linked long chain fatty acids of the diacylphosphatides and the fatty acid esterified in the 2 position of alkyl and alk-1'-enyl glyceryl ethers. For comparison purposes the composition of phosphatidylethanolamine with the inclusion of the aldehydogenic chains of the plasmalogens as acyl is also shown. In addition this table includes the composition of the fatty acids esterified in the 2 position of pig kidney triglycerides, phosphatidylethanolamine and phosphatidylcholine, and the composition of the long chain fatty acids esterified to cholesterol isolated from the pig kidney.

About 73% of the acyl groups esterified to the 2 position in the pig kidney triglycerides are saturated acids. This distribution in this tissue of saturated and unsaturated long chain acids of the triglyceride has been noted previously by Blank and Privett (4). The distribution of the acyl chains of the phosphatides is, however, reversed with respect to their positional specificity compared to that of the triglyceride. This is in accord with the normally seen distribution of fatty acids in the two positions of mammalian diacyl phosphatides (25). The predominant saturated fatty acid of the 2 position of the triglycerides is palmitic acid whereas about 76% of the long chain fatty acids esterified to the 2 position of phosphatidylethanolamine is arachidonic acid. The 2 position of phosphatidylethanolamine is 98% unsaturated while the same position of phosphatidylcholine is about 81% unsaturated. In this phosphatide about 28% of the long chain fatty acids esterified in the 2 position is arachidonic acid while this acid comprises only 1% of the triglyceride acids.

It is possible to calculate the distribution of the fatty acids of triglycerides and polar lipids (with or without the aldehydogenic chain of the plasmalogens as acyl) as described by Mattson and Lutton (26) and Vander Wall (27). Such calculations show that 80% of the palmitic acid in the triglycerides from pig kidney is esterified in the 2 position while only 3% of this chain in phosphatidyl ethanolamine (including the aldehydogenic chain as acyl) is in the same position. Although the biological validity of calculations of this sort has been challenged by Blank and Privett (4), it is quite clear that there is an "inversion" of the structure between triglycerides and polar lipids. Similar calculations further indicate that there is a considerable difference in the distribution of palmitic acid in the 1 position of diacylphosphatidyl ethanolamine from pig kidney and the corresponding chains of vinyl ether linked chains of alk-1'-enyl-acyl phosphatidyl ethanolamine. In *Clostridium butyricum* the composition of these chains is remarkably similar (28).

Metabolically there are several explanations for the "inversion" of the structure of triglycerides and phospholipids (29). Assuming a common diglyceride precursor for triglycerides and polar lipids, it is possible that specific de-acylation and re-acylation occur of the polar lipids (29). Phosphatidyl acyl-cholesterol transacylase (30) seems unlikely to be a major factor in the "inversion" (Table III) unless there is rapid turnover of the resulting cholesteryl ester. The data presented point rather to the danger of translating compositional studies into metabolic relationships between lipid classes. In view of the recent observations (31,32) of intracellular phospholipid exchange, compositional studies of cell organelles may be equally misleading in metabolic terms.

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Stereospecific Analysis of Triglycerides[1]

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ABSTRACT

Stereospecific analysis determines how the fatty acids of triglycerides are distributed over the three different positions of the glycerol. The special problem is the differentiation of position 1-1 and L-3 of glycerol. In the presently known methods, triglycerides are first degraded to mixtures of diglycerides, either by the action of a lipase or by degradation with a Grignard reagent. The isomeric diglycerides are then resolved with the help of a stereospecific enzyme, either a diglyceride kinase or (after conversion of the diglycerides to phospholipids) a phospholipase. It is then possible to analyze or calculate the fatty acid composition for each position on the glycerol. The key to a successful stereospecific analysis is the preparation of a representative diglyceride mixture by a truly random degradation of the triglyceride. The Grignard degradation is the most reliable method, but it is not always applicable, and it is accompanied by some isomerization of glycerides. There is room for improvement in the method. Analyses of natural fats have shown most of them to be asymmetric, i.e., the composition of fatty acids in position 1 differs markedly from that of position 3. Several rules of fatty acid distribution have become apparent.

INTRODUCTION

Since the first stereospecific analysis of a natural triglyceride mixture six years ago (1) methodology has advanced to the extent that determination of the fatty acid distribution of most triglycerides is now possible, with only a few exceptions. Many triglycerides have been analyzed, and some general rules for the positional distribution of fatty acids in fats have become apparent.

This review recapitulates the problem and its nomenclature, and the principles of separating and analyzing optical isomers as applied to triglycerides, concentrating especially on the methods developed in our own laboratory. Then follows a discussion of the critical step in all procedures, the preparation of "representative" diglycerides. The results so far obtained by stereospecific analysis are then reviewed, and finally some remaining problems of the methodology and possible future developments are discussed.

[1]Presented at the 62nd Annual AOCS Meeting, Houston, May 1971.

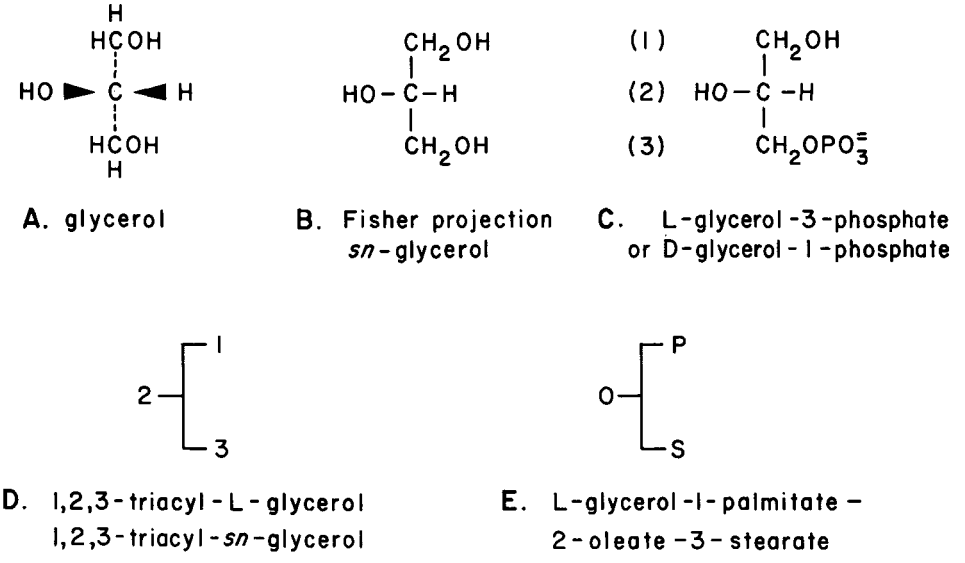

FIG. 1. Structure and nomenclature of glycerol and its derivatives.

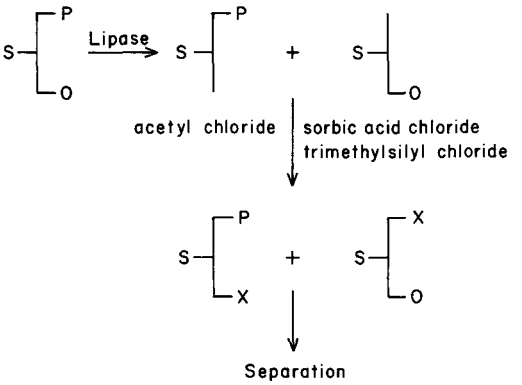

FIG. 2. Configuration of a triglyceride determined according to Morris or Coleman (10,11).

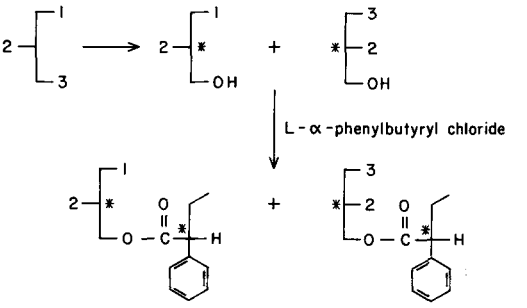

FIG. 3. Conversion of a triglyceride to two diastereomers.

THE PROBLEM AND ITS NOMENCLATURE

The glycerol molecule itself has a plane of symmetry, but when the two primary hydroxyl groups become esterified with different acids the resulting glyceride will be asymmetric or "optically active" (Fig. 1) as will any compound of the substitution pattern *C a b c c* by substitution of one *c*, or of both *c*'s with different substituents. The asymmetrical glycerides can be described without ambiguity by the conventional D, L nomenclature. According to its Fischer projection and the rules of carbohydrate nomenclature (2,3) the compound of Figure 1, C is L-glycerol-3-phosphate; this is the same as D-glycerol-1-phosphate as can be demonstrated by the (permissible) 180° flat rotation of the formula. Consequently, Figure 1, D, in which the numbers stand for fatty acids, is 1,2,3-triacyl-L-glycerol. Figure 1, E gives a specific example of an asymmetric triglyceride.

An alternative system of nomenclature (4), now used by many biochemists dealing with glycerides,originated because it was felt that the D,L system is not able or appropriate to describe the stereochemistry of glycerol and its derivatives. In this system glycerol is stereospecifically numbered (*sn*-glycerol) from top to bottom in the L-form of its Fischer projection (Fig. 1,B) and this numbering is always retained. It can be seen that *sn*-glycerol and L-glycerol are interchangeable in all formulas. Possible objections against the *sn* symbol are its break from established nomenclature, its claim of a special status for glycerol, its needless rigidity, and especially its redundancy. Nevertheless it is being adopted by many lipid biochemists and seems to supplant the D,L-system.

The problem of stereospecific analysis is to determine the fatty acids in each of the three positions of glycerol, and in particular to differentiate the fatty acid in position 1 from that of position 3. (The fatty acid of position 2 can be analyzed with the help of pancreatic lipase or by deacylation with a Grignard reagent.) If in a triglyceride the fatty acids in positions 1 and 3 change places, a triglyceride with identical properties results, except that it is the optical antipode of the first one. The optical rotation of these stereoisomers is usually too small to be measured as was shown by Baer and Fischer for synthetic isomers (5). The problem then is to determine the configuration of a triglyceride by some other means.

The analysis of optical antipodes can take three directions—the physical, the chemical, or the enzymatic—all three initiated by Pasteur (6) who separated the isomeric tartaric acids by differential crystallization, by combination with an optically active base, and by fermentative degradation. The stereospecific analysis of a triglyceride can also be approached from three directions.

In the following the term "stereospecific analysis" is used somewhat ambiguously for two kinds of analyses, but the context will always make clear which is meant. The first is the analysis of the statistical positional distribution of fatty acids in a triglyceride mixture or a natural fat. This analysis will not normally yield much information on the quantity, or even presence or absence, of individual isomers of triglycerides. The other is the analysis of the configuration of a triacid (or diacid) triglyceride, to determine whether it is one of the six possible isomers or a mixture of two or more, i.e., the analysis of a single "species" of triglyceride. This analysis usually requires the preliminary isolation of the species. Otherwise the procedures are identical.

PHYSICAL METHODS OF ANALYSIS

The physical methods have been thoroughly

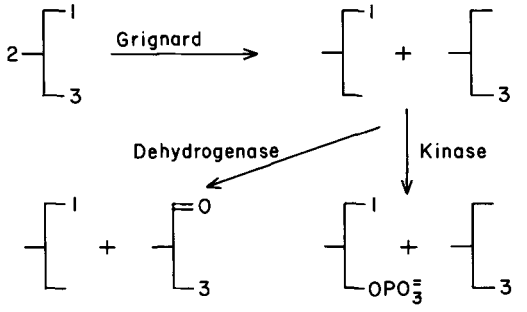

FIG. 4. Hypothetical stereospecific analyses via monoglycerides.

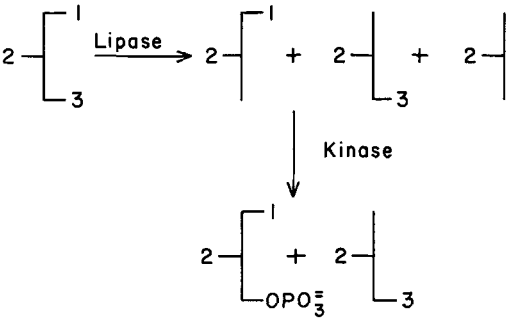

FIG. 5. Stereoanalysis of a triglyceride, Lands and Slakey (17,18).

explored by Schlenk (7) who found that triglycerides in which the three acids differ greatly in chain lengths may show measurable rotation. Later Kleiman et al. (8) actually found, in a seed oil, a monoacetotriglyceride that was optically active; the acetic acid was bound in position 3. Other methods tested by Schlenk require the isolation of a triglyceride species and its comparison with isomers of known configuration. They include the measurement of piezoelectric effect, melting point depression, and x-ray diffraction. In the first reported (1960) "stereospecific analysis" of a triglyceride species, Schlenk examined 1-palmitoyl-2-oleoyl-3-stearoyl glycerol isolated from cocoa and found it to be a racemate (9).

CHEMICAL METHODS

For a chemical analysis of optically isomeric triglycerides two methods have been proposed, one by Morris (10) and one by Coleman (11) (Fig. 2). In both the triglyceride is degraded to a mixture of diglycerides with pancreatic lipase, and the diglycerides are acylated with an acid that will, if it is asymmetrically bound, confer a measurable rotation on its triglyceride. Morris used sorbic acid or trimethylsilylation; Coleman proposed acetic acid. If the resulting triglycerides can be separated their rotation can be measured and the configuration of the original triglyceride can be inferred. In Figure 2 it is assumed that a 1-palmitoyl-2-stearoyl-3-oleoyl-L-glycerol is to be analyzed. The derived triglycerides L-PSX and L-XSO, in which X may be sorbic or acetic acid or the trimethylsilyl group, can be separated according to their unsaturation by silver nitrate chromatography. They will have opposite rotations of about equal magnitude. If the starting triglyceride was L-OSP the signs of rotation of PSX and XSO will be reversed. Since the diglycerides also exhibit measurable optical rotation their acylation is not really essential, but it prevents acyl migration during the separation.

Morris has analyzed several isolated triglyceride species in this manner (12). He found a preponderance of the isomer L-SSO in lard and L-OOS in palm oil, malabar tallow and cocoa butter. Thus the respective triglyceride species in these fats are not racemic mixtures.

The general chemical method of resolving a racemate, introduced by Pasteur, involves its combination with another optically active compound. This results in the formation of diastereomers which are no longer mirror images and which differ in many physical properties. Thus their separation is possible. This method has not yet been applied to triglycerides and may not in fact be applicable, but the principle should be illustrated (Fig. 3). From triglycerides, diastereomers could be prepared by reacting the diglycerides obtained by lipase hydrolysis with an optically active acid, say, L-α-phenylbutyric acid. From a single acid triglyceride, two diastereomeric triglycerides would result; from a natural fat, two sets of diastereomeric triglycerides. In principle these could be resolved; however their separation by presently available methods seems unlikely. Those properties by which triglycerides can be separated—solubility, polarity, complex formation, volatility—are to such a degree functions of the fatty acid chains that they are not likely to be influenced much by changes in the asymmetric centers of the molecule. Perhaps if a more polar constituent, e.g., an optically active amino acid, could be introduced the outlook for a separation of the diastereoisomers by some chromatographic method would improve. The problem is an intriguing one for a chemist; on the other hand, it is of somewhat academic interest because any chemical method is unlikely to offer an improvement over already existing enzymatic methods.

ENZYMATIC METHODS

The resolution of a racemate by an enzyme

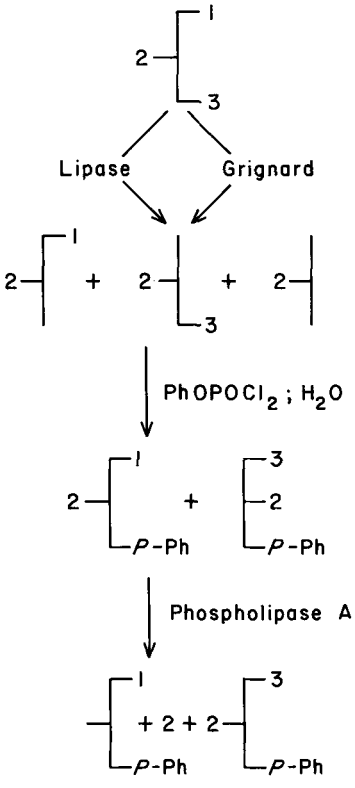

FIG. 6. Stereospecific analysis, first method, Brockerhoff (1).

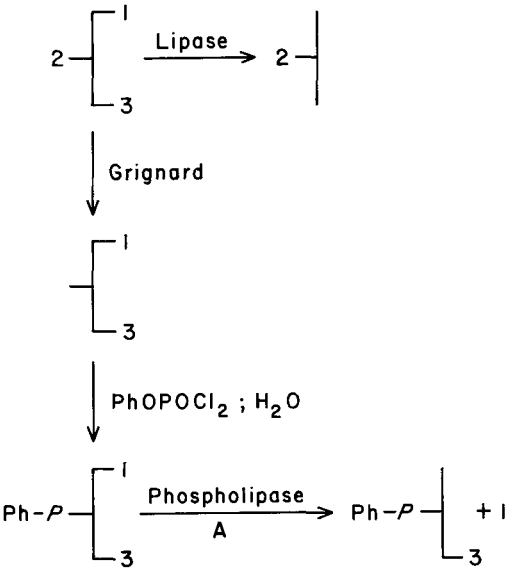

FIG. 7. Stereospecific analysis, second method, Brockerhoff (21).

varies from the general chemical method in that the optically active reagent is the enzyme. Only one of the diastereomers is usually formed and this undergoes a reaction. As a result one isomer of a racemate remains unchanged while its optical antipode is converted into a different compound. The separation is usually not difficult.

The stereoanalysis of a triglyceride would be a very simple matter if an enzyme could be found that would react stereospecifically with triglycerides and hydrolyze one ester bond only, e.g., the bond in position 3 (13). Such an enzyme has not yet been discovered. The lack of stereospecificity of lipases may be surprising because stereospecificity is generally the hall mark of enzymes. Why are lipases an exception, whereas phospholipases are properly stereospecific? Several explanations are possible. One explanation in terms of evolution might be that organisms use lipases to obtain free fatty acids for the synthesis of other lipids or as a source of energy, and the position in which the fatty acids were originally bound to a triglyceride does not matter. Furthermore triglycerides have no structural functions in cells as do phospholipids. They occur in isolated droplets rather than as essential parts of biological membranes, and their configuration, or that of their breakdown products, is of little concern to the organism. This does not imply a lack of species-specific or organ-specific triglycerides of nonrandom steric structure—indeed we know that they exist—but when they are broken down by lipases their structure becomes irrelevant. There has therefore been no need for organisms to develop stereospecific lipases. An explanation in molecular terms is that while the specificity of phospholipases results from the binding of the enzyme to the phosphate group in the natural L-glycerol-3 configuration, no such binding is necessary or possible for lipases. Pancreatic lipase, for instance, requires only a single ester group that is hydrophobic, activated and unhindered (14-16), and other lipases are probably similar. There is little hope that a stereospecific lipase exists.

Since racemic triglycerides themselves cannot be enzymatically resolved, their conversion into products that can be resolved is necessary. Obvious candidates are diglycerides or monoglycerides. Methods using derived 1- and 3-monoglycerides could be developed since fairly representative monoglycerides can now be prepared by Grignard deacylation (Fig. 4). The 1-acyl-L-glycerol might be phosphorylated by a stereospecific monoglyceride or glycerol kinase, or the 3-acyl isomer oxidized by a glycerol dehydrogenase. The appropriate enzymes might still have to be found. It would be worthwhile

TABLE I

Relative Rates of Hydrolysis of Eluoroethyl or Chlorobenzyl Esters of Unsaturated Fatty Acids by Pancreatic Lipase

Acid	Relative rates
Oleic, 9-18:1	1.00
5-14:1	0.13
5-20:1	0.08
5,8,11,14-20:4	0.09
4,7,10,13,16,19-22:6	0.3
5,8,11,14,17-20:5	0.1

to look into the possibilities suggested in Figure 4, because the scheme allows the *direct* analysis of fatty acids 1 and 3 with a minimum of reactions.

The three existing methods are based on the resolution of diglycerides. In the method of Lands and coworkers (17,18) the 1,2- and 2,3-diglycerides are prepared with pancreatic lipase, and the 1,2-diglyceride is phosphorylated by a stereospecific diglyceride kinase of bacterial origin (Fig. 4). The resulting L-phosphatidic acid is separated from the unreacted 2,3-diglyceride and analyzed. Fatty acid 2 is analyzed in the 2-monoglyceride that is also a product of lipolysis, and the composition of fatty acids 1 and 3 is obtained by subtraction.

The method is recommended by its directness and the small number of compounds to be separated and analyzed. It needs truly "representative" diglycerides; this aspect will be discussed later. A shortcoming is that neither fatty acid 1 nor 3 is analyzed directly, but obtained by calculation and therefore subject to cumulative errors of gas chromatographic analysis. The phosphorylation of the 1,2 isomer should be quantitative if the remaining 2,3 isomers are to be useful for the calculation of fatty acid 3. In general a synthesizing enzyme is somewhat less likely to effect a quantitative reaction than is a catabolic enzyme. In its original form the Lands method presents fewer possibilities of checking the accuracy of the results than the two following methods, and it has been less frequently used (17-20).

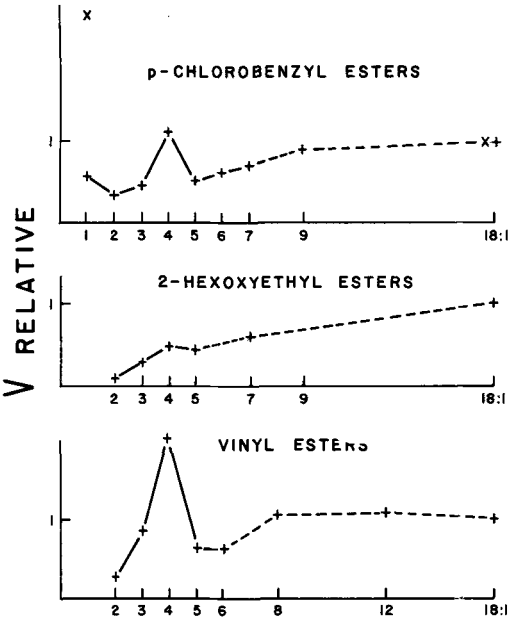

FIG. 8. Lipolysis rates of esters of acids with different chain lengths (16); oleic acid ester=1.

In both methods developed in our laboratory we begin with the diglycerides obtained from the triglyceride with lipase or by deacylation with a Grignard reagent. In the first method (Fig. 6) (1) the mixture of 1,2- and 2,3-diglycerides is isolated by thin layer chromatography (GLC) and converted to an artifi-

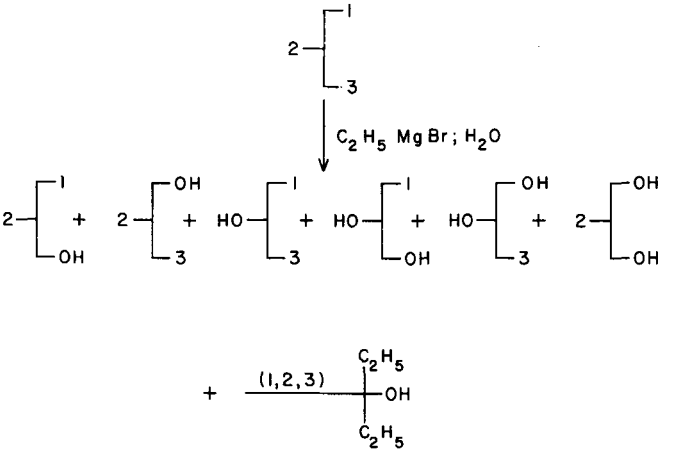

FIG. 9. Deacylation of a triglyceride with a Grignard reagent (31).

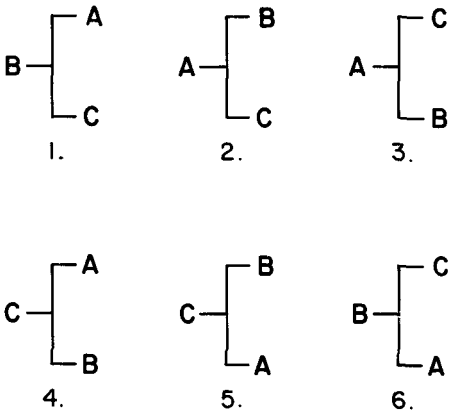

FIG. 10. Isomers of a triacid triglyceride.

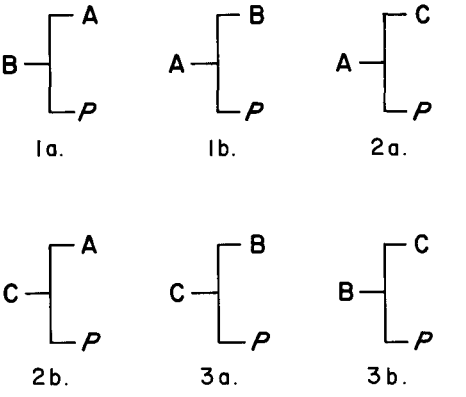

FIG. 11. Isomeric pairs of phosphatidic acids from a triacid triglyceride, Lands and Slakey (33).

cial racemic phospholipid, D- and L-phosphatidyl phenol. Phospholipase A from snake venom attacks only the L-isomer and forms free fatty acid from position 2 and an L-lysophosphatidyl phenol with the fatty acid 1. The remaining D-phosphatidyl phenol can also be analyzed. By subtraction of 2 the fatty acid composition of 3 can be obtained. Fatty acid 2 is also analyzed as the 2-monoglyceride obtained with pancreatic lipase, and subtraction of fatty acids 2 and 1 (the lysophospholipid) from the original triglyceride yields another calculation of fatty acid 3. It is an important feature of the method that fatty acids 1 and 2 can be directly analyzed, fatty acid 2 in two different products, and that the composition of fatty acid 3 can be calculated in two independent ways. In a good analysis the values obtained should of course agree closely; thus the method provides its own control of accuracy. For any analysis to be published it should be ascertained that this control has been used to verify the accuracy of the results.

The second method (21) uses the 1,3-diglyceride that can be isolated after the deacylation of a triglyceride with a Grignard reagent (Fig. 7). It is converted into a phospholipid and this L-2-phosphatidyl phenol is attacked asymmetrically by phospholipase A. Fatty acid 1 is split off; the lysophospholipid contains fatty acid 3. It can be seen that the fatty acids in each position can be directly analyzed. This would make second method the more desirable one if it were not for some difficulty in the preparation of a "representative" 1,3-diglyceride. The correctness of the analysis according to the second method can easily be ascertained by adding the analyses of positions 1,2 and 3; they must total the fatty acid composition of the triglyceride.

Most steps in both the first and the second method pose no difficulties. Synthesis of the phospholipids and their enzymatic hydrolysis can be carried to completion. Separation of the different products is achieved by TLC on silicic acid. The 1,3-diglyceride which is a product of the Grignard deacylation is more easily isolated if *ethyl* magnesium bromide is employed (22) instead of the methyl magnesium bromide originally used, because the tertiary alcohols that are formed from the fatty acids are less polar if they contain ethyl groups, and move farther away from the 1,3-diglycerides on the thin layer plate. Oxidation of intermediates is a problem with polyunsaturated triglycerides; it can be alleviated by working under nitrogen and adding antioxidants to all solvents (22,23). The truly difficult step in both methods (as well as in that of Lands and Slakey) is the preparation of representative diglycerides.

PREPARATION OF DIGLYCERIDES

A stereospecific analysis can succeed only if the fatty acids of all intermediates are representative of the fatty acids of the original triglycerides in the different position. In other words none of the reaction steps in the procedure must select intermediates with certain fatty acid compositions and discriminate against others. The chemical phosphorylation and the deacylation with phospholipase A fulfill this condition since the reactions are quantitative; but the deacylation of the starting triglyceride by lipase yields at best around 30% diglycerides because the diglycerides are themselves degraded by lipase; and pancreatic lipase does discriminate against glycerides with certain fatty acids (24,25). Table I shows its relative reaction rates against esters of some naturally occurring unsaturated acids (14,26). It can be seen that the pentaenoic and hexaenoic acids which are prominent in fish oils are quite

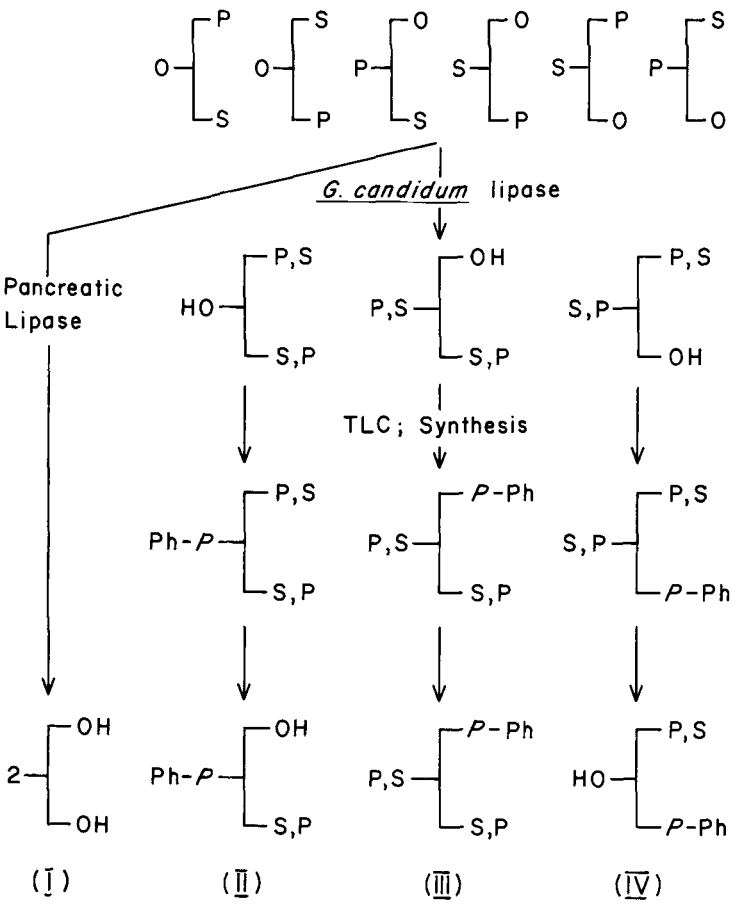

FIG. 12. Determination of the isomers in a monounsaturated triacid triglyceride, Sampugna and Jensen (30).

resistant against lipase. Figure 8 shows reaction rates for esters of acids with different chain lengths (14). Of the short chain fatty acids, butyric acid is split fastest by the lipase; this makes the analysis of milk fats of ruminants problematic. Nevertheless reasonably representative diglycerides have been prepared from milk fat in two laboratories (27,28). Marine oils however do not yield representative diglycerides with lipase (26,29).

Sampugna and Jensen (30) have used a lipase from a microorganism *Geotrichum candidum* which splits, from triglycerides, fatty acids with a *cis* double bond at carbon 9. Thus the use of pancreatic lipase can be avoided. In special cases this method has other advantages which will be discussed below.

The analysis of marine oils became possible when Yurkowski and Brockerhoff (31) treated triglycerides with a Grignard reagent (Figure 9). All other deacylation methods attempted were accompanied by the migration of acyl groups in the glycerides, but from the Grignard reaction truly representative racemic 1,2-diglycerides could be isolated with a yield between 15-25%. Furthermore 1,3-diglycerides were also obtained, making our second method of stereoanalysis possible. However while the 1,2-diglycerides had the required composition, the 1,3-diglycerides were found to be 6-10% contaminated with what was originally 1,2-diglyceride (31,22). Acyl migration had taken place. This reduces the accuracy of the second method. At present the best general procedure of stereoanalysis begins with the racemic 1,2-diglycerides obtained by Grignard deacylation followed by the first method.

DETERMINATION OF THE ISOMERS IN A TRIACID TRIGLYCERIDE

A triglyceride species with three different fatty acids can occur in six isomeric forms (Fig. 10). Stereoanalysis as outlined so far can give

the fatty acid composition in each of the positions, but not the proportions of the six isomers. For instance if 30% of the acid in position 1 is fatty acid A, then isomer 1 and isomer 4 together constitute 30% of the total triglyceride, but their relative proportion is unknown. In isomer 1, position 2 contains fatty acid B and position 3 contains C, but the B of position 2 is shared by isomers 1 and 6, and the C in 3 by isomers 1 and 2, and again the proportions remain unknown. The proportion of isomers in one pair of triglycerides must be determined before the others can be calculated. The problem was recognized by Schlenk (7) and later further discussed (32); solutions were proposed by Lands and Slakey (33) and by Jensen et al. (34). Both solutions recognized that a separation of isomeric pairs can be achieved at the di-acid level. For instance one of the isomeric pairs of phosphatidic acids in Lands's method (Fig. 11), e.g., 1a plus 1b, can be isolated by chromatography and then digested with phospholipase A. In the pair 1a and 1b of Figure 11 the ratio of fatty acids A and B in position 2, as analyzed with the phospholipase, gives the proportion of isomers 1a and 1b and therefore the proportion of the triglycerides ABC and BAC. The procedure of Jensen et al. (34) can be applied if only one of the fatty acids of a triacid triglyceride is *cis*-Δ^9 unsaturated. This acid may be split off by the lipase of *G. candidum*. Sampugna and Jensen (30) have developed a cheme of stereoanalysis based on the specificity of this lipase (Fig. 12). It can be seen that each of the phospholipids at the bottom of the scheme represents a pair of isomeric triglycerides, namely the pair in which oleic acid occupied the position now occupied by the phenol phosphate group. A fatty acid determination of compound II will yield the ratio of the first two triglyceride isomers.

POSITIONAL DISTRIBUTION OF FATTY ACIDS IN FATS

To date most stereoanalyses have been carried out on natural fats, i.e., on mixtures of many triglycerides. The results of such analyses can be represented as in Table II (35) which shows the average concentrations of fatty acids in the three positions, in mole per cent. Such an analysis shows the over-all stereospecific distribution of fatty acids or the tendency of each fatty acid to occupy one of the three positions; however it does not give the proportions of individual isomers. If these are to be determined the fat has first to be fractionated into triglyceride species (or small groups of species). So far only one complete analysis of a

TABLE II

Fatty Acid Distribution in Animal Depot Fats

Animal	Position	Fatty acid, mole %								
		14:0	16:0	16:1	18:0	18:1	18:2	20:1	22:1	20:5	22:5	22:6
Rabbit	1	3	34	9	6	25	14					
	2	6	25	12	1	26	23					
	3	1	24	7	3	35	22					
Pig	1	2	16	3	21	44	12					
	2	4	59	4	3	17	8					
	3	<1	2	3	10	65	24					
Cod	1	6	15	14	6	28	2	12	6	2	1	1
	2	8	16	12	1	9	2	7	5	12	3	20
	3	4	7	14	1	23	2	17	7	13	1	6

TABLE III

Monounsaturated Triglycerides in Cocoa Butter[a]

Acid	Mole %	Acid	Mole %
POP	12.1	SOS	24.2
L-SSO	0.8	L-OPP	0.0
L-OSS	0.7	L-PPO	1.1
L-PSO	0.3	L-OPS	0.0
L-OSP	0.0	L-SPO	2.1
L-SOP	16.3	L-POS	16.5

[a]Sampugna and Jensen (30).

triglyceride fraction, the composition of the monounsaturated fraction of cocoa butter, has been reported (Sampugna and Jensen) (36) (Table III). It can be seen that the major diacid triglycerides POP and SOS are symmetrical, and the major triacid triglyceride is racemic SOP. Such a high degree of symmetry in triglycerides is probably exceptional; it seems that in this case no difference is made between palmitic and stearic acid during biosynthesis. If *oleic* acid occupies an α position, three out of the four identified triglycerides are nonracemic (Table III).

Since the separation of fats into single triglyceride species is exceedingly laborious, some workers have separated families of triglycerides according to their degree of unsaturation, by silver nitrate chromatography, and stereoanalyzed these families separately. This is a compromise giving more information than an analysis of the total fat; in particular it permits some judgments on the accuracy of certain theories of fatty acid distribution. The results can be presented in quantities of triglyceride fractions such as SMD, DMS and the like, where S, M and D stand for saturated, monoenoic and dienoic acid respectively. These values may then be compared with those obtained on the basis of different distribution theories (18,37-41).

The stereoanalyses of unfractionated fats have supplied the most information on the general distribution patterns of fatty acids in triglycerides. First it has become clear that symmetrical fatty acid patterns or completely racemic triglycerides must be the exception, not the rule. A completely racemic triglyceride has so far only been found in cocoa butter (36), and it is also possible that the depot fats of some or many birds are racemic (43,44) although egg yolk triglyceride is asymmetric (42). One species of fish, a trout, was also found to have a symmetrical pattern (44). The only analyses of triglycerides of microorganisms showed highly asymmetrical distributions (45).

TRIGLYCERIDES OF PLANTS

Relatively few stereospecific analyses of vegetable oils have been published (46-49). Apparently the fatty acid distribution is more symmetrical in plants than in animals but is in no case yet found completely racemic (Table IV), although major triglyceride species such as the SOP of cocoa butter may be racemates. Any general rules of distribution cannot be drawn from the present data except the well-known one (50) that saturated acids, and acids longer than C_{18}, occupy the 1 and 3 positions. The stereospecific distribution of acids may in fact differ in different strains of the same species (39,51,52). This effect appears to be under genetic control.

In three vegetable fats with anomalous fatty acids these were found entirely in position 3:acetic acid in the seed oils of *Euonymus verrucosus* (8) and of *Impatiens edgeworthi* (47), and an allenic estolide acid in *Sapium sebiferum* (48).

MILK FATS

The milk fat of ruminants contains large

TABLE IV

Positional Distribution of Fatty Acids in Vegetable Fats (46)

Vegetable fat	Position	Fatty acids, mole %						
		16:0	18:0	18:1	18:2	18:3	20:1	22:1
Rapeseed	1	4	2	23	11	6	16	35
	2	1	—	37	36	20	2	4
	3	4	3	17	4	3	17	51
Corn	1	18	3	28	50	1		
	2	2	—	27	70	1		
	3	14	3	31	52	1		
Olive	1	13	3	72	10	1		
	2	1	—	83	14	1		
	3	17	4	74	5	1		

TABLE V

Positional Distribution of Fatty Acids in a Fraction of Bovine Milk Triglycerides[a]

Position	4:0	6:0	8:0	10:0	12:0	14:0	16:0	18:0	18:1
1	—	—	—	1	11	11	41	15	20
2	—	—	—	4	23	4	37	4	12
3	53	24	6	5	—	—	5	2	7

[a]Breckenridge and Kuksis (53).

amounts of short chain fatty acids, especially butyric acid. Stereospecific analyses have been carried out by Pitas et al. (17) and Breckenridge and Kukis (28,53); it has been found that the butyric acid is almost exclusively bound to position 3 (Table V). In the milk fats of ruminants (27,28,53), man (38) and whales (54), but not of sheep and goat (55), palmitic acid prefers the 2 position as in lard.

DEPOT FATS OF ANIMALS

It is possible to deduce some general rules of positional fatty acid distribution from the large number of analyses performed. These rules are anything but rigid; rather they are guidelines. They describe well the distribution of a majority of fatty acids in a majority of fats, although they are everywhere punctuated by partial exceptions. I have found it helpful to arrange the experimental values as in Figure 13, in which each fatty acid is plotted according to its proportion found in the respective position (43). The pattern for the rabbit is typical for mammalian fats. Saturated acids tend toward position 1, shortchain acids and unsaturated fatty acids toward position 2. Position 3 seems to attract longer chain fatty acids, and the composition is nearer to random, i.e., 33%, than in the other positions. Pig fat is anomalous in that it has most of the palmitic acid in position 2. The relative position of 16:0 to 16:1 should be noticed.

In cod liver oil (Fig. 13) the short chain and the polyunsaturated fatty acids accumulate in position 2 as does palmitic acid. The "normal" pattern of 16:1 vs. 16:0 is again reversed, as in lard. The sequences of fatty acids in the plots of Figure 13 are reminiscent of the so-called "reverse phase" chromatograms, especially in positions 1 and 2. This indicates that fatty acid distribution depends on distribution of the acids between polar and nonpolar phases. Possibly the "specificity of the acylating enzymes" can in part be explained on this basis (43,56).

GENERAL RULES OF FATTY ACID DISTRIBUTION

The typical animal fat may be formulated as in Table VI. This formula describes no more than the general positional *tendencies* of fatty acids.

In the pig and in most (but no all) fish, and also in human, cetacean and bovine milk (but not in goat and sheep milk) we have superimposed on this pattern a preference of palmitic acid, 16:0, for the 2 position. In the

TABLE VI

Tendencies of Positional Distribution of Fatty Acids in Depot Fats

Fat	Position	Fatty acid
Animals	1	Saturated
	2	Short, unsaturated
	3	Long, random
Pig, most fish, (milk)	2	16:0
Birds	1,3	Symmetrical (?) (body fat)
Mammals	3	20:5, 22:5, 22:6
Milk (ruminants)	3	4:0, 6:0
Plants	1	Saturated, long
	2	Unsaturated (18:2)
	3	Saturated, long
Plants	3	Acetic acid, estolide acids
All fats	3	"Strange" acids

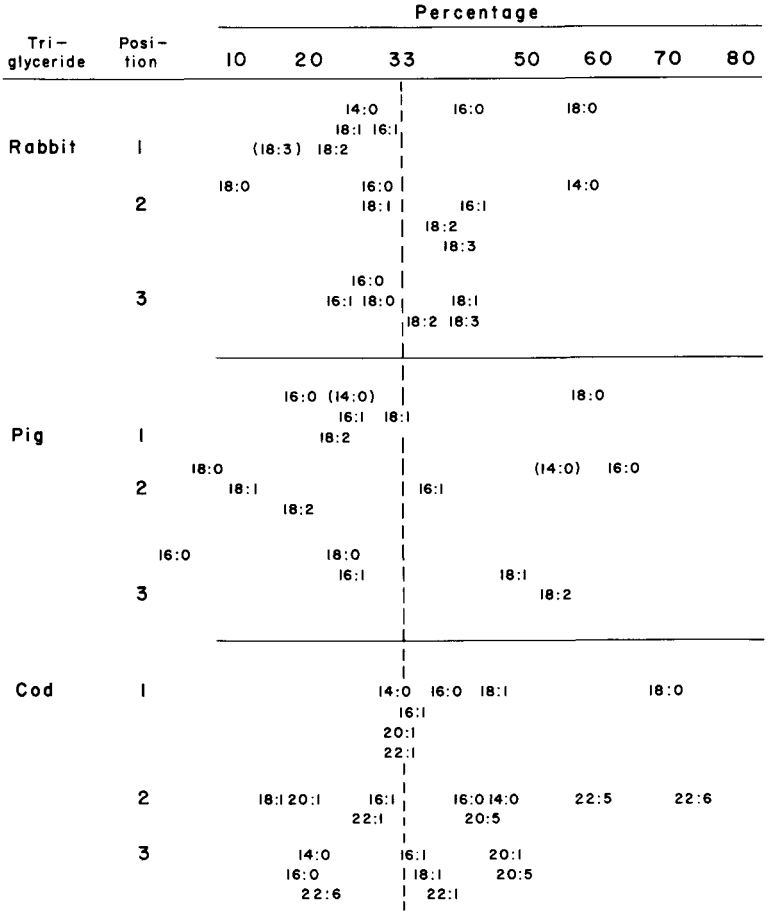

FIG. 13. Proportional positional distribution of fatty acids (43,44).

depot fats of seals and whales we also find an accumulation of 16:0 in position 2, but there the general pattern is still obeyed and 16:0 is pushed into position 2 only because the primary positions are occupied by C_{20} and C_{22} fatty acids (43). There is proportionally more 16:1 and much more 14:0 in the 2 position in seals and whales. These fats clearly do not belong to the same group as those of pig and fish. The positional distribution of palmitic acid divides animal fats into two distinct groups, and it seems that two different biochemical pathways may be the cause for this division.

The depot fats of birds (Table VI) may be supposed to be more symmetrical than those of mammals (as long as there are no analyses to contradict this statement). Invertebrates seem to follow the general animal pattern though somewhat more indistinctly.

If the fats of mammals contain long chain polyenoic acids such as 20:5, 22:5 or 22:6, i.e., if the animals feed on a diet of marine origin, these acids are predominantly in position 3 (23,57), whereas in fish or invertebrates they prefer position 2 according to the general formula (44). More logically, in mammals, position 2 is barred for these polyenoic acids. The distribution between 1 and 3 then follows the general formula. Litchfield (58) has found

TABLE VII

Positional Percentage Distribution of Long Chain Polyenoic Acids in Depot Fats[a]

	22:5, 22:6	
Position	Fish, invertebrates	Mammals
1	9	31
2	69	7
3	22	61

[a]Litchfield (58).

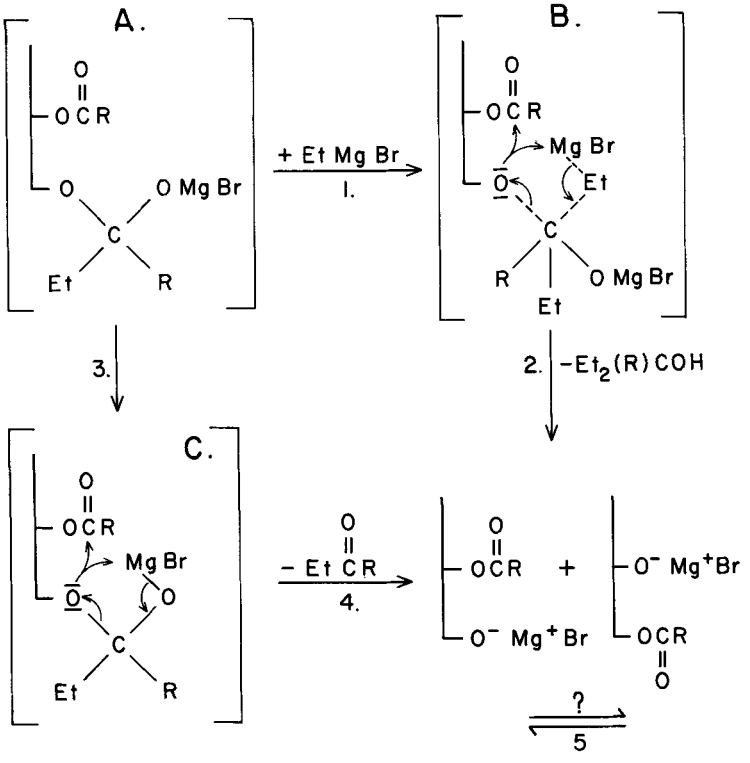

FIG. 14. Hypothetical mechanisms of acyl migration during Grignard deacylation.

that the distribution of 22:5 and 22:6 can in each case be closely predicted (Table VII). In fish and invertebrates two-thirds of the acids are in position 2; of the rest, two-thirds are in position 3 and one-third in position 1. In marine mammals less than one-tenth is in position 2; of the bulk, two-thirds are in 3 and one-third in 1.

In milk fat of ruminants, butyric and hexanoic acid are in position 3 (Table VI).

In plants (Table VI) we find saturated acids and long chain monoenoic ($>C_{18}$) acids almost exclusively in positions 1 and 3; this rule was already formulated by Mattson and Volpenhein (50). There are no distinct general rules of steric distribution except for those fats that have exceptional fatty acids, as acetic acid or an estolide acid.

We can postulate the general rule for all fats that any "strange" fatty acid is likely to be esterified to position 3, "strange" fatty acids being those not found in the common range of C_{12} to C_{22} acids, e.g., acetic, butyric, hexanoic or estolide acids, or not regularly found in the depot fats of a particular group of organisms, e.g., the long chain polyenoic acids in mammals. This rule is very plausible for biochemical reasons. Positions 1 and 2 are acylated by enzymes specific for phospholipids, in particular phosphatidic acid (59), but position 3 is acylated at the diglyceride stage, and we are reminded of the different specificities of the catabolic enzymes, i.e., the much stricter specificities of phospholipases as compared to lipases. It seems likely that the diglyceride acylases are also much less demanding as to the structure of the substrates than the lysophosphatidic acid acylases; or, to put it differently, if a "strange" acid must be incorporated it is done with minimal upheaval of the system if it is done as the last step.

STEREOSPECIFIC ANALYSIS IN METABOLIC STUDIES

Stereospecific analysis has been applied in metabolic studies to follow the pathway of a fatty acid into a certain position of glycerol, or to detect the influence of an agent, or diet, on triglyceride synthesis. We determined that the marine polyenoic acids will accumulate in position 3 not only in marine, but also terrestrial, mammals (57), and Christie and Moore (41) studied the influence of dietary copper on

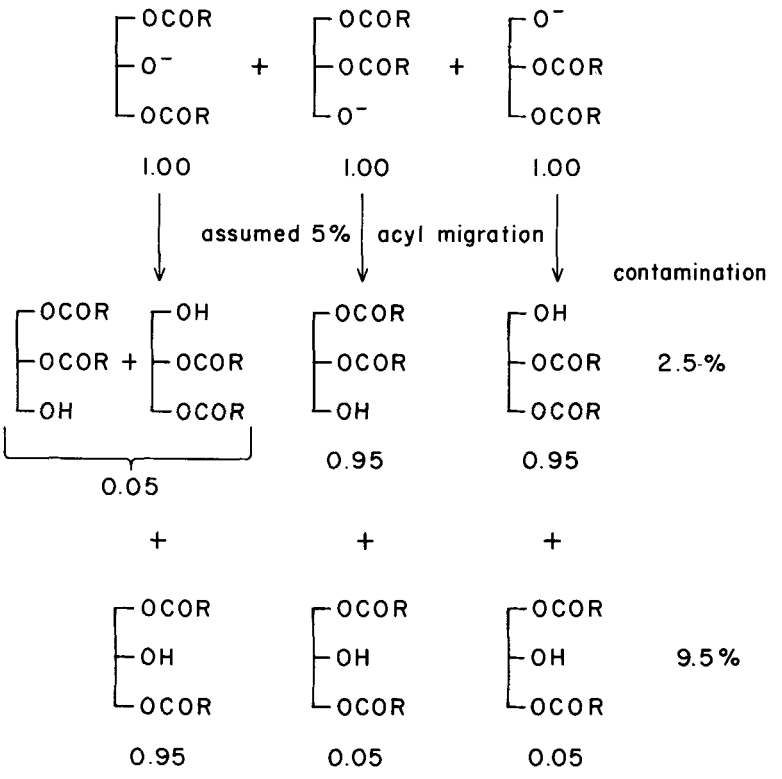

FIG. 15. Cross-contamination of diglycerides obtained by Grignard deacylation.

pig triglycerides. Snyder and Wood found that irradiation of bone marrow (60) and tumor formation in rats (61,62) both change the asymmetric fatty acid distribution of the triglycerides.

Stereospecific analysis is also able to determine the positional distribution of a radioactive label in a triglyceride, and the method has been used by Akesson, Elovson and Arvidson to investigate the bioacylation of diglycerides (63-65).

POSITIONALLY RESTRICTED RANDOM DISTRIBUTION

Lipid chemists have always been tempted to search for a simple mathematical formula to describe the distribution of fatty acids between triglyceride molecules and between positions on the glycerol. The theory of random distribution, which stated that the fatty acids were esterified randomly over all hydroxyl groups, proved incorrect when analyses with lipase showed that fatty acid compositions in positions 1 plus 3 and position 2 were always different. It was supplanted by the 1,3-random-2-random theory (66-68). We know now that the compositions in positions 1 and 3 are also different; this brings us to the 1-random-2-random-3-random theory which states that fatty acids are selective as to positions on the glycerol but are, within these positions, evenly distributed over all triglyceride molecules. This implies that during the biosynthesis of glycerides the fatty acid or acids that are already in a glyceride molecule do not influence the selective acylation of the remaining position or positions. Lands and Slakey (18) speak of "noncorrelative" acylation. The percentage of each isomer of a fat can then be calculated by simply multiplying the concentrations of the fatty acids in the three positions. Conversely if the concentration of a single isomer or a group of isomers is known, it can be used to verify the theory. This has been done for rat liver triglycerides (18) and pig liver (37) and the results agreed with the theory. For egg yolk triglycerides the agreement was also good, though there was evidence for some selectivity of acylation in certain triglyceride species (42). Analyses of a variety of pig tissues gave the same result (69) except in the case of the blood triglycerides where the discrepancies with the theory were great. On the other hand, correlative specificity has been found in the only

vegetable oil investigated, corn oil (39,51,52).

If we assume that "noncorrelative" biochemical acylation is the rule, the 1-random-2-random-3-random rule will still be applicable only if a fat has been synthesized in one tissue under similar circumstances. However if it is a mixture from several sources, e.g., endogenous and exogenous, or different tissues, or from one tissue but synthesized at different times and with changed diets, there will be a nonrandom distribution of the positionally restricted fatty acids between glycerides. The position-restricted-random-distribution hypothesis should not therefore be accepted as a general rule, though it may hold approximately for a majority of fats.

PROBLEMS AND PROGNOSIS OF THE METHODOLOGY

The Grignard deacylation has made most triglycerides accessible to stereoanalysis, but some difficulties remain. Although butter fat has been successfully analyzed via the pancreatic lipase route it is doubtful, in view of the chain length specificity of the lipase, if isolated triglyceride species of butter could be analyzed in this way. The Grignard deacylation of butter, on the other hand, leads to a mixture of long,long-1,2-diglycerides and long,short-1,3-diglycerides which has not yet been separated (31).

Difficulties may also be expected with those marine fats containing isovaleric acid, which is very resistant against lipase; with the Grignard deacylation the same problem as that encountered with butter fat would prevail.

Fats with conjugated double bonds seem to react, at this site, with Grignard reagents (C. Litchfield, personal communication); they may also resist lipase degradation (14).

It would obviously be desirable to use a method that directly isolates the different positions, without resource to calculations. Such a method exists in the second procedure developed in our laboratory; however the critical intermediate, the 1,3-diglyceride, cannot be prepared without contamination with perhaps 10% of isomerized 1,2-diglycerides (31,22). This makes the method, at this time, less accurate than our first method.

No thorough study has yet been made of the mechanism of the Grignard deacylation and of the problem of acyl migration during this procedure. I suggest the mechanisms shown in Figure 14. The first intermediate is the addition product of one molecule of a Grignard reagent to a carboxyl group. This intermediate could decompose to a diglyceride and a ketone (steps 3 and 4), although the formation of ketones has not yet been observed in triglyceride deacylation. Alternatively the first intermediate could add another Grignard molecule (step 1) and eliminate a tertiary alcohol (step 2). In either case a negatively charged, nucleophilic oxygen appears on the glycerol and can be immediately neutralized by the magnesium bromide ion, or the reagent can first attack the neighboring carboxyl group, with acyl migration as a result. The two possibilities are illustrated with the forked arrows in the formulas B and C.

It is also possible that the diglycerides finally formed, which are initially present as alcoholates of magnesium, can isomerize (step 5) as long as the reaction is not stopped with water or acid.

To obtain uncontaminated, representative 1,3-diglycerides, measures should be found that prevent the acyl migration. If migration occurs at step 5, shortening of the reaction time should depress it, as should lowering of the temperature. Shielding the nucleophilic oxygen might depress migration in reactions 1-2, 2-3 or 5.

In experiments to test these possibilities (unpublished) I found that reaction time and temperature have little influence on acyl migration. Temperatures from -20 to +60 C and reaction times from 15-300 seconds all yielded 1,3-diglyceride with 6-10% contamination. I conclude that reaction 5, the isomerization of the diglycerides, is not of major importance and that acyl migration occurs mainly at the rearrangement of the transition compounds B or C.

Shielding the nucleophilic oxygen of B or C might be accomplished with a polar solvent. The choice of solvents is severely restricted because the Grignard reagent will react with all fairly polar compounds. Only halogenated hydrocarbons are sufficiently resistant yet slightly more polar than the diethyl ether used in the original deacylation procedure. I tested 2-chloropropane, dichloromethylene and p-fluorotoluene, with disappointing results: the 1,3-diglycerides still showed 6-10% contamination. With nonpolar solvents, benzene or hexane, the yield of 1,3-diglyceride became very small.

It is somewhat surprising that while the 1,3-diglyceride is contaminated with isomerized 1,2-diglyceride, the 1,2-diglyceride that is formed by the Grignard deacylation appears to be completely "representative." An explanation is given in Figure 15. Random deacylation yields twice as much DL-1,2-diglyceride as 1,3-diglyceride, and if acyl migration takes place to the same degree in all compounds, the

relative contamination of the 1,3-diglyceride will be almost four times greater.

The chemical deacylation of triglycerides deserves further study. Completely representative 1,3-diglycerides should be prepared, and perhaps also monoglycerides. It may be necessary to abandon the magnesium-Grignard compounds and look for different reagents.

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SHORT COMMUNICATIONS

Enzymatic Conversion of 5α-Cholest-8(14)-en-3β-ol to 5α-Cholesta-8,14-dien-3β-ol

ABSTRACT

[3α-^{3}H]-Cholest-8(14)-en-3β-ol was incubated with a 10,000 x g supernatant fraction of a rat liver homogenate. The enzymatic formation of labeled cholesta-8,14-dien-3β-ol was indicated by the results of studies employing column chromatographic and cocrystallization techniques.

INTRODUCTION

Cholest-8(14)-en-3β-ol has been shown to be convertible to cholesterol in rat liver homogenate preparations (1,2). The same sterol has been isolated from rat skin (2,3). The mechanisms involved in the enzymatic conversion of cholest-8(14)-en-3β-ol to cholesterol are incompletely understood at this time. The convertibility of cholest-8(14)-en-3β-ol to cholest-7-en-3β-ol in rat liver homogenate preparations incubated under aerobic conditions has been demonstrated (2). While the efficient conversion of cholest-8-en-3β-ol to cholest-7-en-3β-ol can readily be demonstrated under anaerobic conditions (4,5), no detectable conversion of cholest-8(14)-en-3β-ol to cholest-8-en-3β-ol or cholest-7-en-3β-ol could be demonstrated under these conditions (2). This combination of findings suggested the possibility of an oxygen dependent step in the conversion of cholest-8(14)-en-3β-ol to cholest-7-en-3β-ol. We have suggested the possibility of a 15-hydroxy derivative as a potential intermediate in the over-all process and have demonstrated the convertibility of the two epimers (at C-15) of cholest-8(14)-en-3β,15-diol to cholesterol in rat liver homogenate preparations (6). The results of recent investigations have also provided evidence suggesting a possible intermediary role of $\Delta^{8,14}$-sterols in the biosynthesis of cholesterol (7-12).

The purpose of the present communication is to present evidence indicating the formation of cholesta-8,14-dien-3β-ol from cholest-8(14)-en-3β-ol in rat liver homogenate preparations.

MATERIALS AND METHODS

The chemical synthesis and purities of cholest-8(14)-en-3β-ol, cholesta-8,14-dien-3β-ol, [3α-^{3}H]-cholest-8(14)-en-3β-ol, and [3α-^{3}H]-cholesta-7,14-dien-3β-ol have been described elsewhere (2,5,8,13). Procedures used for the measurement of radioactivity, colorimetric assay of sterols, the preparation of the 10,000 x g supernatant fraction of rat liver homogenates, and the separation of sterols on

TABLE I

Cocrystallization of Labeled Material Derived From [3α-^{3}H]-Cholest-8(14)-en-3β-ol[a] With Authentic Cholesta-8,14-dien-3β-ol

Material	Specific activity of crystals, dpm/mg	Specific activity of mother liquor, dpm/mg
Initial	954 ± 29	
After recrystallization from methanol	504 ± 15	3960 ± 20
After second recrystallization from methanol	469 ± 9	549 ± 5
After third recrystallization from methanol	456 ± 9	459 ± 9
After recrystallization from acetone water	462 ± 14	447 ± 22
After second recrystallization from acetone water	469 ± 14	449 ± 22

[a]Cochromatographed with cholesta-8,14-dien-3β-ol.

TABLE II

Crystallization of Cholesta-8,14-dien-3β-ol in the Presence of [3α-^{3}H]-Cholesta-7,14-dien-3β-ol

Material	Specific activity of crystals, dpm/mg	Specific activity of mother liquor, dpm/mg
Initial	11,600 ± 117	
After recrystallization from methanol	2130 ± 38	36,800 ± 257
After second recrystallization from methanol	1280 ± 26	4490 ± 68

columns of alumina-Super Cel-silver nitrate have been described previously (14,15).

EXPERIMENTAL PROCEDURE AND RESULTS

[3α-^{3}H]-Cholest-8(14)-en-3β-ol (75 μg; 1.32 x 10^6 cpm) in propylene glycol (0.2 ml) was incubated with 50 ml of a 10,000 x g supernatant fraction of rat liver homogenate for 3 hr at 37 C as described previously (14). The sterols were recovered from the saponified incubation mixture by extraction with petroleum ether and applied to an alumina-Super Cel-Silver nitrate column (50 x 1 cm) (14,15) along with unlabeled cholest-8(14)-en-3β-ol (4.5 mg), cholesterol (2.3 mg), and cholesta-8,14-dien-3β-ol (3.1 mg). Using a mixture of chloroform-acetone 98:2 as the eluting solvent, fractions 5.5 ml in volume were collected. Aliquots were taken for measurement of radioactivity and colorimetric assay of sterol content. Most (ca. 87%) of the recovered radioactivity was eluted in fractions 7-11 and had the same chromatographic mobility as that of the incubated substrate. Approximately 9% of the activity was associated chromatographically with cholesterol (fractions 17-26). A small but significant amount of radioactivity (3.5%) was eluted as a discrete peak in fractions 39-57, corresponding to the mobility of authentic cholesta-8,14-dien-3β-ol. It is important to note that, while complete resolution of cholesta-8,14-dien-3β-ol from 7-dehydrocholesterol and cholesterol (and other monounsaturated sterols) is realized in this chromatographic method, cholesta-8,14-dien-3β-ol and cholesta-7,14-dien-3β-ol are not well resolved in this system. The contents of fractions 39-57 were pooled and diluted with authentic, unlabeled cholesta-8,14-dien-3β-ol and the resulting material was subjected to repeated crystallization. After one recrystallization from methanol, the specific activity of the crystals decreased to ca. 53% of the initial value. After a second crystallization from methanol, the specific activity of the crystals was ca. 49% of the initial value. Further recrystallization from methanol and acetone-water did not result in a significant change in the specific activity of the crystals or of the mother liquor (Table I).

That cocrystallization represents a useful criterion for distinguishing between cholesta-8,14-dien-3β-ol and cholesta-7,14-dien-3β-ol is indicated by the results of the following experiment. [3α-^{3}H]-Cholesta-7,14-dien-3β-ol (1.47 x 10^5 dpm; 5 μg) was diluted with unlabeled cholesta-8,14-dien-3β-ol (15 mg) and the resulting mixture was recrystallized twice from methanol. After the first recrystallization the specific activity of the crystals decreased to ca. 22% of the initial value. After the second recrystallization, the specific activity of the crystals was ca. 11% of the initial value (Table II).

Upon chromatographic analysis of the non-saponifiable material recovered after incubation of [3α-^{3}H]-choles-8(14)-en-3β-ol with a homogenate preparation which has been heated at 100 C for 30 min prior to the addition of substrate, no radioactivity was associated with cholesterol or cholesta-8,14-dien-3β-ol.

DISCUSSION

The finding that cholest-8(14)-en-3β-ol is convertible to cholesta-8,14-dien-3β-ol in rat liver homogenate preparations is of importance in consideration of possible mechanisms involved in the enzymatic conversion of cholest-8(14)-en-3β-ol to cholesterol. Moreover, since experimental evidence exists (summarized in Reference 2) which suggests a possible intermediary role, albeit not necessarily obligatory, of $\Delta^{8(14)}$-sterols in the biosynthesis of cholesterol, the present findings are also of importance in consideration of possible intermediates in the enzymatic conversion of various $\Delta^{8(14)}$-sterols to cholesterol in animal tissue.

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In Vivo Studies on Ganglioside Metabolism

The following pathway has been proposed for the biosynthesis of gangliosides based principally on a series of reactions catalyzed by embryonic chick brain particles (1):

Ceramide + UDP-glu→ceramide-glu

ceramide-glu + UDP-gal→ceramide-glu-gal

ceramide-glu-gal + CMP-NANA→ ceramide-glu-gal-NANA (G_{M3})

ceramide-glu-gal-NANA + UDP-gal NAc→ ceramide-glu-gal-(NANA)-gal NAc (G_{M2})

ceramide-glu-gal(NANA)-gal NAc + UDP-gal→ ceramide-glu-gal (NANA)-gal-NAc-gal (G_{M1})

Several of these individual reactions have been detected in other tissues (2,3,4,5,6). In vivo studies designed to establish precursor-product relationships involved in the synthesis of gangliosides have been largely unsuccessful. This is largely due to the nonspecificity of radioactive precursors previously employed, which has included serine-^{14}C (7), glucose-^{14}C (7,8,9,10,11), galactose-^{14}C (7,8), acetate-^{14}C (12) and glucosamine-^{14}C (7). Recent publications have indicated that N-acetyl-^{3}H mannosamine specifically labels only the sialic acid moities of gangliosides in vivo (13). The present study was undertaken to elucidate the metabolic interrelationships of the individual gangliosides after the administration of N-acetyl-^{3}H mannosamine to young rats.

Acetic anhydride-^{3}H was purchased from ICN-Tracerlab and mannosamine-HCl from Pfanstheil. Silica Gel G plates, 0.5 mm thickness, were obtained from Analtech.

N-Acetyl-^{3}H mannosamine was synthesized according to a published procedure (14) and shown to contain only one radioactive product by paper chromatography. The final specific activity was 1.587×10^{8} dpm/μ mole N-acetyl mannosamine.

Sprague-Dawley rats, 15 days old, were injected intercerebrally with 3.4×10^{8} dpm of N-acetyl-^{3}H mannosamine in 10 μl of water. Groups of five animals were sacrificed at 30 min, 1 hr, 2 hr, 4 hr, 8 hr and 24 hr. The brains were removed, pooled and the mixed gangliosides were isolated as previously described (15). The ganglioside mixture was fractionated into individual components by thin layer chromatography employing chloroform-methanol-2.5 N NH_4OH (60:35:8) as the solvent (16) and a typical result is presented in Figure 1. Individual spots were visualized by brief iodine exposure and the bands of silica gel corresponding to standards were removed from the plates and weighed. Aliquots were counted with a thixotropic gel (17) in a Packard scintillation counter for determination of radioactivity.

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In Vivo Studies on Ganglioside Metabolism

The following pathway has been proposed for the biosynthesis of gangliosides based principally on a series of reactions catalyzed by embryonic chick brain particles (1):

Ceramide + UDP-glu→ceramide-glu

ceramide-glu + UDP-gal→ceramide-glu-gal

ceramide-glu-gal + CMP-NANA→ ceramide-glu-gal-NANA (G_{M3})

ceramide-glu-gal-NANA + UDP-gal NAc→ ceramide-glu-gal-(NANA)-gal NAc (G_{M2})

ceramide-glu-gal(NANA)-gal NAc + UDP-gal→ ceramide-glu-gal (NANA)-gal-NAc-gal (G_{M1})

Several of these individual reactions have been detected in other tissues (2,3,4,5,6). In vivo studies designed to establish precursor-product relationships involved in the synthesis of gangliosides have been largely unsuccessful. This is largely due to the nonspecificity of radioactive precursors previously employed, which has included serine-^{14}C (7), glucose-^{14}C (7,8,9,10,11), galactose-^{14}C (7,8), acetate-^{14}C (12) and glucosamine-^{14}C (7). Recent publications have indicated that N-acetyl-^{3}H mannosamine specifically labels only the sialic acid moities of gangliosides in vivo (13). The present study was undertaken to elucidate the metabolic interrelationships of the individual gangliosides after the administration of N-acetyl-^{3}H mannosamine to young rats.

Acetic anhydride-^{3}H was purchased from ICN-Tracerlab and mannosamine-HCl from Pfanstheil. Silica Gel G plates, 0.5 mm thickness, were obtained from Analtech.

N-Acetyl-^{3}H mannosamine was synthesized according to a published procedure (14) and shown to contain only one radioactive product by paper chromatography. The final specific activity was 1.587×10^{8} dpm/μ mole N-acetyl mannosamine.

Sprague-Dawley rats, 15 days old, were injected intercerebrally with 3.4×10^{8} dpm of N-acetyl-^{3}H mannosamine in 10 μl of water. Groups of five animals were sacrificed at 30 min, 1 hr, 2 hr, 4 hr, 8 hr and 24 hr. The brains were removed, pooled and the mixed gangliosides were isolated as previously described (15). The ganglioside mixture was fractionated into individual components by thin layer chromatography employing chloroform-methanol-2.5 N NH_4OH (60:35:8) as the solvent (16) and a typical result is presented in Figure 1. Individual spots were visualized by brief iodine exposure and the bands of silica gel corresponding to standards were removed from the plates and weighed. Aliquots were counted with a thixotropic gel (17) in a Packard scintillation counter for determination of radioactivity.

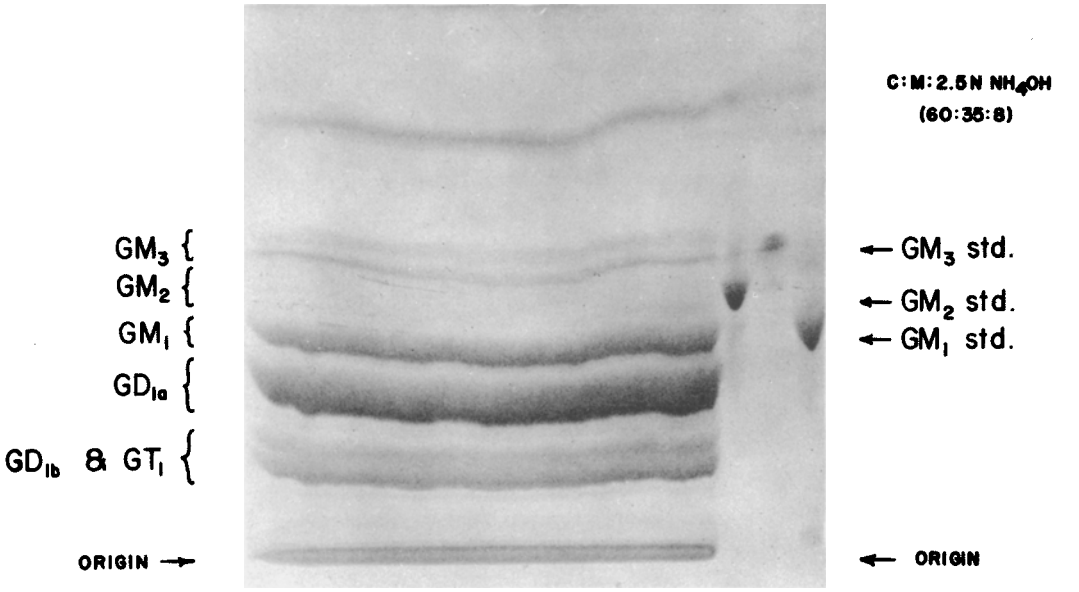

FIG. 1. Thin layer chromatogram of the ganglioside fraction from 15-day-old rat brains. Details are provided in the text.

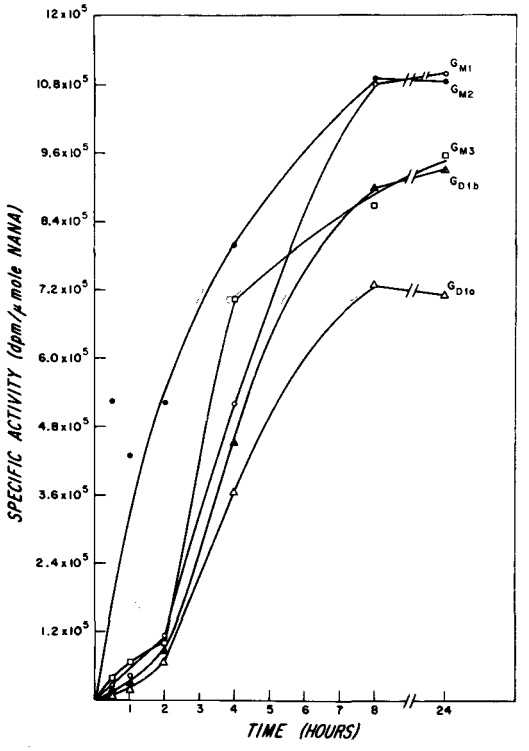

FIG. 2. Specific activity of the individual gangliosides as a function of time after the intercerebral administration of N-acetyl-^{3}H mannosamine to 15-day-old rats. The curve labeled GD_{1b} represents the mixed GD_{1b} and GT_1 area on the plate in Figure 1.

Another weighed quantity of the gel was hydrolyzed with 0.05 N H_2SO_4 for 60 min at 80 C. The sialic acid liberated was quantitated by the colorimetric procedure of Aminoff (18). The specific activity was then calculated from the total radioactivity and sialic acid content of each ganglioside fraction.

The increase in specific activity as a function of time for the individual gangliosides is presented in Figure 2. It is readily apparent that, except for G_{M2}, all the gangliosides show a lag period of almost 2 hr before appreciable label is present. A very rapid incorporation of the radioactive precursor into G_{M2} is seen without this initial lag period. The highest specific activity was found in both G_{M2} and G_{M1}, a value which was attained at 8 hr. It is significant that G_{M3}, a postulated precursor of the higher ganglioside homologues, has a 2 hr lag period and had a specific activity lower than either G_{M2} or G_{M1}. Two pertinent observations of other investigators suggest that G_{M3} may not be a precursor of the other gangliosides. The majority of naturally occurring lipids are characterized by a heterogenous fatty acid composition; however 80-90% of the fatty acid in brain gangliosides is reported to be stearic acid (19). The fatty acid composition of brain G_{M3} has been found to contain only 30-40% of stearic acid (20). Tay-Sachs disease is an "inborn metabolic" error characterized by the accumulation of G_{M2} and a dimunition of hexosaminase "A" (21). A neutral sphingolipid,

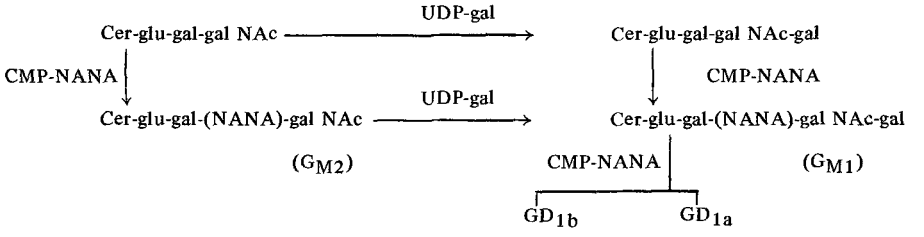

FIG. 3.

ceramide-glu-gal-gal NAc, also accumulates in this disease (20,22). There is little, if any, increase in the G_{M3} levels in pathological material (20). These three lines of evidence would tend to mitigate against a precursor role of G_{M3} for the other brain gangliosides. The glycosphingolipid composition of mouse neuroblastoma and human and rat glioma cells grown in tissue culture has been reported (23). G_{M3} was the only ganglioside present in the gliomas. In the neuroblastoma cells G_{M3} was undetectable, while G_{M2} and its corresponding asialo derivatives were abundant. They concluded, therefore, that G_{M3} does not serve as a precursor of the higher ganglioside homologues (23).

Two possible pathways are explained by Figure 3.

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Determination of the Position of Ethylenic Linkages in Lipids

ABSTRACT

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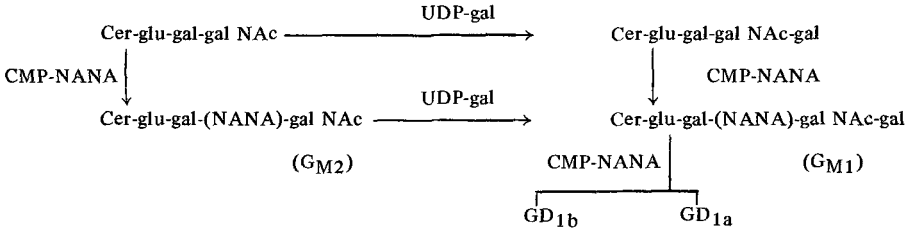

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The same products were obtained on subjecting methyl *trans*-9,10-epoxyoctadecanoate to the above procedure.

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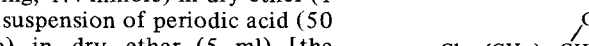

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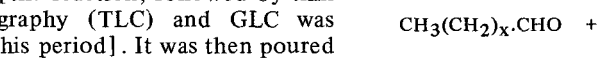

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[Received June 24, 1971]

Ricinoleic Acid in *Linum mucronatum* Seed Oil

ABSTRACT

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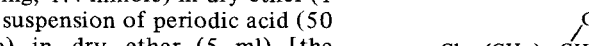

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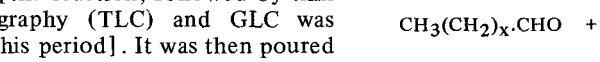

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Ricinoleic Acid in *Linum mucronatum* Seed Oil

ABSTRACT

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in the Linaceae. Identification was made by IR, gas liquid chromatography, nuclear magnetic resonance, ozonolysis and mass spectrometry. Other major components of the oil are oleic acid (24%) and linoleic acid (48%).

Although ricinoleic acid has been found in small amounts in many seed oils (1), especially in the genus *Lesquerella* (2), its major source is castor oil. Seed from the genus *Linum*, though extensively surveyed by Yermanos et al. (3), has not previously been known to produce ricinoleic acid. We now report its presence in seeds of *Linum mucronatum* Bertol. collected in Turkey.

IR spectra (Perkin-Elmer 137) of the petroleum ether extract of *L. mucronatum* seeds exhibited a sharp band at 2.8 μ indicative of free hydroxyl groups. Thin layer chromatography (TLC) on a 0.25 mm layer of Silica Gel G showed a spot that migrated as a monohydroxy triglyceride. Methyl esters prepared (4) from this oil contained 15% of a component that behaved like a hydroxy ester on both gas liquid chromatography (GLC) and TLC. Equivalent chain lengths (ECL) (5) were determined from Apiezon L and LAC-2-R446 columns in a Packard Model 7401 gas chromatograph at 190 C. The unknown component had ELC's of 19.6 from Apiezon L and 24.7 from LAC-2-R446, which are similar to those found for methyl ricinoleate by Miwa et al. (5). In addition to normal esters, TLC showed a major component with migration characteristics similar to those of monohydroxy esters.

The hydroxy ester was isolated by TLC on 1 mm layers of Silica Gel G. IR of the isolated ester again showed the hydroxyl band and the absence of any *trans* unsaturation (10.4 μ). The nuclear magnetic resonance (Varian HA-100) spectrum was consistent with that of a hydroxy ester with one double bond. Ozonolysis-GLC (6) established the double bond at the 9,10 position.

A portion of the hydroxy ester was hydrogenated in ethanol with palladium on charcoal as catalyst. GLC-mass spectrometry [Dupont (CEC) 21-492-1 with jet enricher system] of the trimethylsilyl derivative of saturated hydroxy ester gave major ions with masses of 301 and 187 in agreement with fragments expected to arise from a 12-trimethylsilyoxy group; namely, $H_3CO_2C(CH_2)_{10}CHOSi(CH_3)_3^+$ and $CH_3(CH_2)_5CHOSi(CH_3)_3^+$. A prominent peak at m/e=371 represented M (386) - 15, which is usual for trimethylsilane derivatives.

TABLE I

Fatty Acid Composition of *Linum mucronatum* Seed Oil

Component	Area % by gas liquid chromatography
16:0	6.0
18:0	3.6
18:1	24
18:2	48
18:3	2.0
20:1	0.4
Ricinoleic	15
Others, 11	0.8

Optical rotatory dispersion (Cary Model 60) of the unsaturated hydroxy ester in methanol gave a plain positive curve with $[\alpha]_D^{26}$=+5.2 deg and $[\alpha]_{300}^{26}$=+22 deg. These values have the same sign as and, considering the small sample used, agree adequately with those of methyl ricinoleate (7). Therefore the hydroxy ester is methyl(+)-12-D-hydroxy-*cis*-9-octadecenoate. The total fatty acid composition of *L. mucronatum* oil is given in Table I.

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2-Monoglyceride as an Aid to the Absorption of Cholesterol Into the Thoracic Lymph

ABSTRACT

Absorption of radioactive cholesterol given in triolein was nearly three-fold that of a similar dose given in ethyl oleate or oleic acid. The difference appears to reflect a need for monoglyceride, since addition of 2-monoolein to ethyl oleate improved cholesterol absorption.

Recently we reported that cholesterol was equally well absorbed from a variety of fats and oils by lymph duct cannulated rats (1). Of these only hydrogenated coconut oil depressed the absorption rate. In subsequent experiments we have observed that ethyl oleate also depressed cholesterol absorption compared with the triglyceride (Table I).

The experimental conditions employed have been described in detail (1). Male Wistar rats (200-300 g) were given, via stomach cannula, test doses of ^{3}H- or 4-^{14}C-cholesterol in the different lipid media. Lymph was collected over a 24 hr period and a portion was extracted (2) for scintillation counting. Triolein, safflower oil and the labeled cholesterol were from the earlier sources (1). Ethyl linoleate was prepared from safflower oil (3); ethyl oleate from olive oil by transesterification and fractional crystallization from acetone; and oleic acid also from olive oil (4). Oleic acid chloride, prepared from oleic acid (5), was reacted with 1,3-benzylidene glycerol and the benzylidene group was then removed with boric acid (6), to give 2-monoolein.

Absorption of labeled cholesterol from triolein was the same on both days 2 and 3 after surgery (1). It was 3-fold that from an equivalent amount of ethyl oleate (Table I). Absorption from mixtures of ethyl oleate and triolein was inversely related to the amount of ethyl oleate present. Further comparisons revealed that absorption of cholesterol from safflower oil was likewise approximately 3-fold that from ethyl linoleate in 24 hr (Table II). Absorption from ethyl linoleate was essentially equal to that from ethyl oleate and oleic acid. Thus there was no relationship between the degree of unsaturation and amount of cholesterol appearing in the lymph during either 8 or 24 hr periods. It follows that the protective action of linoleate and other unsaturates (7) against hypercholesterolemia is apparently not a direct effect on cholesterol absorption from the intestinal tract, since ethyl linoleate exerts such action and ethyl oleate does not (8).

The striking difference between the triglyceride and the ethyl ester as vehicles for cholesterol absorption appears to be due to the potential of the former to form 2-mono-

TABLE I

Comparison of Ethyl Oleate and Triolein as Media for Intestinal Cholesterol Absorption in the Male Rat[a]

Test oil	Day received	Number of rats	Total radioactive cholesterol (μmoles) in lymph during 0-24 hr
Triolein	2	4	25.7 ± 3.4
Ethyl oleate	2	9	9.1 ± 2.2
Triolein-ethyl oleate 1:2 v/v	3	5	11.8 ± 2.5
Triolein-ethyl oleate 2:1 v/v	2	5	19.8 ± 2.2
Triolein	3	4	24.5 ± 0.6

[a]After overnight fasting, thoracic ducts and stomachs were cannulated using sodium pentobarbital anesthetic (2.5 mg/100 g body weight). Heparin, 125 USP units, was injected intraperitoneally during surgery and about 24 hr later. Cannulated rats, in restraining cages, were allowed free access to water containing 0.64% NaCl and 0.04% KCl. The first day after the operation each rat received 0.8 ml triolein without added cholesterol. The next day each rat was given via stomach cannula 50 μmoles of cholesterol, containing 2.5 μC of ^{3}H-cholesterol, dissolved in 0.8 ml of test oil. The third day 50 μmoles of cholesterol containing 0.5 μC of 4-^{14}C-cholesterol was given in 0.8 ml of a different test oil except in the case of triolein. Cholesterol was extracted from the collected lymph with acetone-ethanol (1:1) and radioactivity was determined by scintillation counting. Values are expressed as mean ± standard error of the mean.

TABLE II

Lymphatic Recovery of Radioactive Cholesterol Following Gastric Administration in Different Forms of Fatty Acids to Male Rats[a]

Test oil	Number of rats	Total radioactive cholesterol (μmoles) in lymph during 0-8 hr	0-24 hr
Safflower oil	7	13.3 ± 2.1	19.6 ± 1.8
Ethyl linoleate	7	3.6 ± 0.3	7.5 ± 0.5
Ethyl oleate	7	3.9 ± 0.5	8.0 ± 1.1
Oleic acid	7	2.8 ± 0.6	7.1 ± 1.7
Ethyl oleate + 2-monoolein, 2:1 mole/mole	6	10.7 ± 0.5	16.6 ± 0.9
Triolein	8	10.2 ± 1.1	19.8 ± 1.4

[a]Rats and lymph samples were treated as described under Table I. Comparisons of the various forms of oleic acid were made with oleic acid mole content comparable to 0.8 ml of triolein. Values are expressed as mean ± standard error of the mean.

glyceride in the digestive tract, since the addition of 2-monoolein to ethyl oleate in a 1:2 molar ratio sharply improved cholesterol absorption. The observation cannot be explained on the basis of a lower rate of hydrolysis of the ethyl ester than the triglyceride by pancreatic lipase (9), since absorption from oleic acid was not better than from the ethyl ester.

While the need for monoglycerides for micelle formation and for the inclusion of cholesterol in micelles in vitro has been clearly demonstrated (10,11), and the implication that this process is fundamental to the absorption of cholesterol has been widely accepted, we believe this is the first direct, in vivo demonstration of the need for 2-monoglyceride in cholesterol absorption.

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Structural Relationships Between Glycerides of Pig Serum and Adipose Tissue

ABSTRACT

Structural analyses have been performed on the triacylglycerols and phosphoglycerides isolated from adipose tissue, serum chylomicrons, and serum lipoproteins from pigs. The triacylglycerols from adipose tissue contained mainly palmitate esterified at the 2 position of the glycerol moiety, whereas those from the serum had predominately

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Ethyl oleate	7	3.9 ± 0.5	8.0 ± 1.1
Oleic acid	7	2.8 ± 0.6	7.1 ± 1.7
Ethyl oleate + 2-monoolein, 2:1 mole/mole	6	10.7 ± 0.5	16.6 ± 0.9
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Structural analyses have been performed on the triacylglycerols and phosphoglycerides isolated from adipose tissue, serum chylomicrons, and serum lipoproteins from pigs. The triacylglycerols from adipose tissue contained mainly palmitate esterified at the 2 position of the glycerol moiety, whereas those from the serum had predominately

TABLE I

Fatty Acid Analyses of Triacylglycerols

Tissue and lipid class	Fatty acid, wt %					
	16:0	18:0	18:1	18:2	18:3	20:4
Adipose						
Triacylglycerol	26.2	16.5	43.6	9.2	0.6	3.8
2-Monoacylglycerol	72.3	3.6	10.7	3.2	—	—
Serum chylomicrons [a]						
Triacylglycerol	16.7	4.3	30.8	43.9	2.9	1.2
2-Monoacylglycerol	11.3	6.6	35.6	40.6	2.9	2.9
Serum (less chylomicrons)						
1st Sample						
Triacylglycerol	14.3	5.9	41.1	34.4	1.5	3.0
2-Monoacylglycerol	11.5	6.2	38.5	37.9	2.1	3.9
2nd Sample						
Triacylglycerol	21.8	7.5	34.0	32.7	2.2	1.6
2-Monoacylglycerol	11.0	5.9	37.1	40.8	3.0	2.3

[a]Pooled from both serum samples.

unsaturated fatty acids esterified at the 2 position. The phosphatidylcholine from the serum and the phosphatidylcholine and phosphatidylethanolamine from the adipose tissue also contained saturated fatty acids at the 1 position and unsaturated fatty acids at the 2 position. The phosphatidylethanolamine fraction from the adipose tissue contained over 50% alk-1-enyl moieties at the 1 position. The unusual triacylglycerol structure found in the adipose tissue of the pig is independent of the circulating glycerides and appears to be formed within the adipose cells by a pathway different from that for the phosphoglycerides.

Since the techniques for the structural analysis of triacylglycerols and phosphoglycerides have been established, it has been shown that for the majority of animal tissues the fatty acid esterified at the 2 position of the glycerol tends to be unsaturated, while that esterified at the 1 position tends to be saturated (1-6). An exception to this generalization is found in the triacylglycerols of pig adipose tissue, which have 60-80% palmitate esterified at the 2 position, while the acids located at the 1 and 3 positions tend to be unsaturated (7-11). The triacylglycerols from kidney, spleen, lung, pancreas and heart of the pig also contain predominately saturated acyl moieties at the 2 position (8,11). In contrast, the triacylglycerols from pig liver (8,11,12) and the phosphoglycerides from the liver and kidney of pigs (13) all have the more common "2-unsaturated" structure. The blood triacylglycerols from pigs show a partial enrichment in the palmitate content at the 2 position, and appear to fall between the two structural extremes (11).

A possible mechanism that could explain the "2-saturated" structure in the pig adipose tissue relates to the uptake of 2-monoacylglycerols derived from "2-saturated" triacylglycerols of chylomicrons, and the de novo synthesis of triacylglycerols in the adipose cells via the monoacylglycerol pathway. This pathway has been demonstrated in hamster adipose tissue (14). The likelihood of this possibility is suggested by the fact that the triacylglycerols in lymph from sheep contain predominately the "2-saturated" structure (15). To test this hypothesis we have investigated the structure of triacylglycerols from serum chylomicrons and lipoproteins of the pig. We also describe the nature of the aliphatic moieties at the 1 and 2 positions of phosphatidylcholine from the serum and the phosphatidylcholine and phosphatidylethanolamine of the adipose tissue from pigs.

Adipose tissue was obtained from the peritoneum of a pig killed at a local slaughterhouse, and the lipids were extracted using the method of Bligh and Dyer (16). Blood samples from pigs were obtained from the University of Tennessee-AEC Agricultural Research Station. The serum chylomicrons were collected by centrifugation (17) and the two samples pooled. The pooled chylomicron fraction and the two serum fractions were extracted with chloroform-methanol 2:1 v/v. Neutral lipids were separated from the phosphoglycerides on silicic acid columns that were eluted first with chloroform and then with methanol. The tri-

TABLE II

Fatty Acids of Phosphoglycerides from Adipose Tissue and Serum of Pigs

Tissue and lipid class	Fatty acid, wt %								
	16:0	16:1	17:0	18:0	18:1	18:2	20:4	22:6	Other
Adipose tissue									
Phosphatidylethanolamine									
1 Position	6.7 (14.1)[a]	1.3		26.5 (29.9)	9.7 (9.6)	1.6			
2 Position	8.6	1.9		5.0	22.3	32.3	24.4	1.3	4.0
Phosphatidylcholine									
1 Position	17.9	1.2	1.6	57.0	18.0	2.7			
2 Position	8.3	1.1	0.4	8.6	15.4	14.4	46.3	3.5	2.0
Serum									
Phosphatidylcholine									
1 Position	69.0	0.8	4.0	20.6	3.9	1.0			
2 Position	14.4	1.2	2.5	11.7	18.0	28.2	7.4	2.5	14.5

[a]Values in parentheses refer to dimethylacetals of aldehydes of same carbon number.

acylglycerols and the individual classes of phosphoglycerides were purified by preparative thin layer chromatography (TLC).

The triacylglycerols were incubated with hog pancreatic lipase (Pierce Chemical Company) (1) for 30 min, and the fatty acids and 2-monoacylglycerols produced were isolated by preparative TLC. Phosphoglycerides were incubated with phospholipase A (*Crotalus adamanteus* venom, Ross Allen's Reptile Institute, Inc.) for 2 hr (18) and the fatty acids and lysophosphatides isolated by TLC. Methyl esters were prepared from each of the lipid samples by heating with 7% HCl in methanol in a sealed tube at 100 C for 15 min. The methyl ester preparations were analyzed by gas liquid chromatography on a column of 10% EGSS-X (Applied Science Labs, Inc.) in a Victoreen chromatograph (Model 4000) equipped with a flame ionization detector.

We found that the triacylglycerols of adipose tissue had a "2-saturated" structure which agrees with previous investigators (7-11), whereas the triacylglycerols of both the serum chylomicrons and the serum lipoproteins had "2-unsaturated" structures (Table I). The phosphoglycerides from the adipose tissue from the pig also have the "2-unsaturated" structure (Table II) in contrast to the triacylglycerols from the same tissue. The high content of dimethylacetals produced from the aliphatic moiety of position 1 of the phosphatidylethanolamine fraction implies that this lipid class is more than half plasmalogen.

Our results demonstrate that the monoacylglycerol pathway cannot account for the unusual "2-saturated" structure of the triacylglycerols of adipose tissue and certain other organs of the pig. Instead it appears that either there is more than one group of enzymes involved in the acylation of glycerophosphate in the synthesis of triacylglycerols and phosphoglycerides in these pig tissues, or that there is extreme specificity of the diacylglycerol acyltransferase and the phosphorylbase diacylglycerol transferases.

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